Porcine models for the study of local and systemic regulation of innate immune factors in obesity

Innate immune gene expression and circulating biomarkers in obesity-induced inflammation

Højbøge, Tina Rødgaard

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PhD thesis

Tina Rødgaard Højbøge

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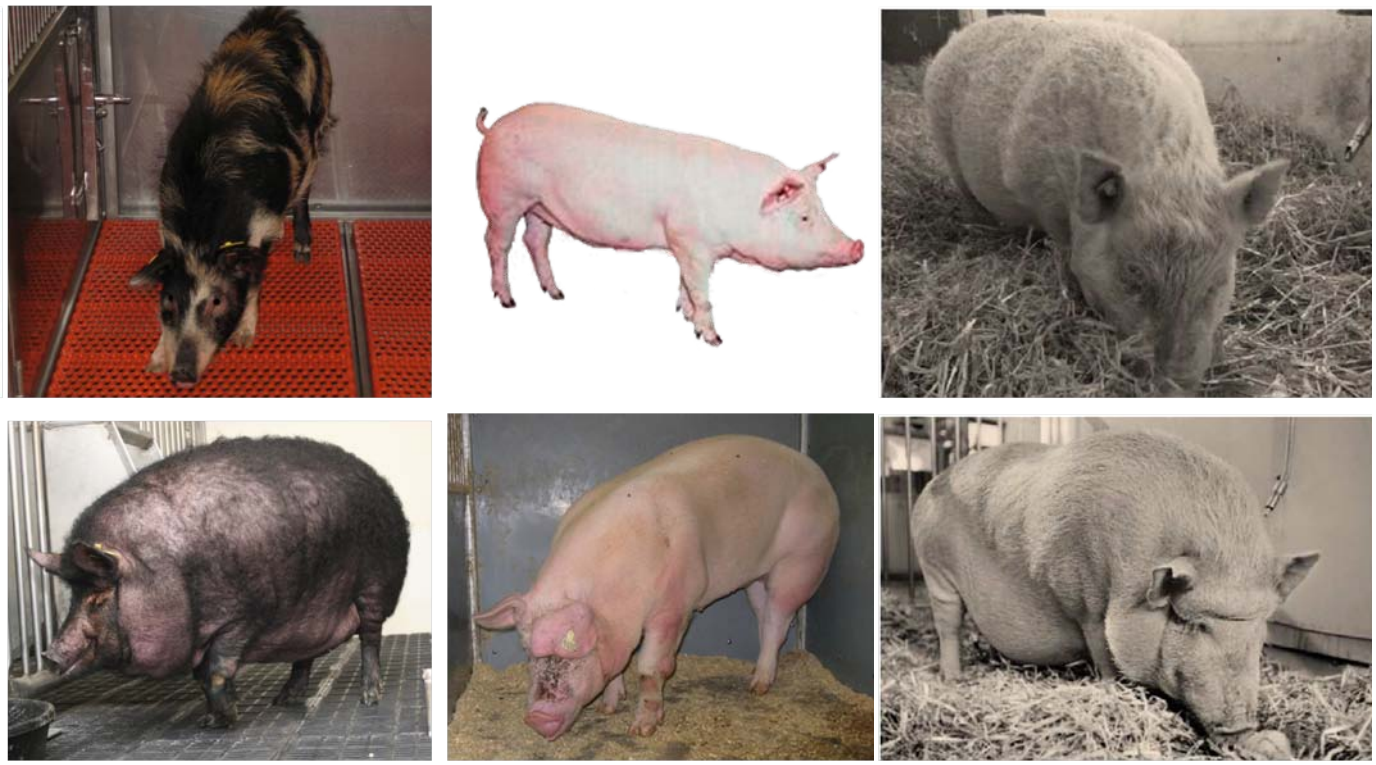

Supervisor:

Professor Peter M. H. Heegaard
Innate Immunology Group
National Veterinary Institute
Technical University of Denmark

Co-supervisor:

Senior Scientist Jan Stagsted
Department of Food Science
Faculty of Sciences and Technology
Aarhus University

Assessment committee:

Professor Karsten Kristiansen
Department of Biology
University of Copenhagen

Research leader Pascale Chavatte-Palmer
Developmental Biology and Reproduction
National Institute of Agronomical Research

Professor Gregers Jungersen (chairman)
National Veterinary Institute
Technical University of Denmark

Cover illustrations: From left to right; lean (top – own picture) and obese (bottom - courtesy of Michael Sturek) Ossabaw minipigs, lean (top - http://www.danbred.com/Products/Mother_breeds.aspx) and obese cloned (bottom - courtesy of Jan Stagsted) Yorkshire/Landrace crossbred (domestic) pigs, lean (top – courtesy of the University of Copenhagen) and obese (bottom – courtesy of Novo Nordisk A/S) Göttingen minipigs.

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Innate immune gene expression and circulating biomarkers in obesity-induced inflammation

Preface

This Ph.D. thesis is based on experimental work performed under the supervision of Professor Peter M. H. Heegaard from National Veterinary Institute, DTU and senior scientist Jan Stagsted, Ph.D., Department of Food Science, Aarhus University. The Ph.D. study was initiated in December 2008 and ended in September 2012. The Ph.D. project was financed partially by funds provided by the National Veterinary Institute, DTU and by a grant from the Danish Strategic Research Council (FØSU 2101-06-0034), as part of a larger project entitled "Nutriomics – functional foods for cloned lean/obese pigs".

The thesis is based on the following papers:

Paper I
Rødgaard, T., Skovgaard, K., Stagsted, J. and Heegaard, P. M. H. (2012) Cellular Reprogramming: 14(5), Epub ahead of print. Expression of Innate Immune Response Genes in Liver and Three Types of Adipose Tissue in Cloned Pigs

Paper II
Rødgaard, T., Skovgaard, K., Stagsted, J. and Heegaard, P. M. H. (2012) Cellular Reprogramming (manuscript submitted). Cloning changes the response to obesity of innate immune factors in blood, liver and adipose tissues in domestic pigs

Paper III
Rødgaard, T., Skovgaard, K., Moesgaard, S. G., Cirera, S., Christoffersen, B. Ø. and Heegaard, P. M. H. (2012) Journal of Animal Science (manuscript submitted). Characterization of gene expression in obese Göttingen minipigs: Extensive gene expression changes in liver and adipose tissue in otherwise well-adapted, obese Göttingen minipigs

Paper IV
Rødgaard, T., Stagsted, J., Christoffersen, B. Ø., Moesgaard, S. G., Cirera, S., Sturek, M., Alloosh, M. and Heegaard, P. M. H. (2012) Veterinary Immunology and Immunopathology (in press). Orosomucoid expression profiles in liver, adipose tissues and serum of lean and obese domestic pigs, Göttingen minipigs and Ossabaw minipigs

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Secondly, this thesis would not have been possible without the help of numerous people.

I wish to thank my supervisor Peter M. H. Heegaard, who is one of the most skilled scientists I have had the pleasure of working with, and whose optimism and insight have sparked many ideas. I really hope we will continue our good collaboration in the future with new projects. Also I wish to thank my co-supervisor Jan Stagsted for his help and support, as well as the external collaborators and co-authors for their contribution to this thesis.

Thank you to my colleagues at DTU-VET for making my time here fun and enjoyable.

I wish to thank Kerstin Skovgaard and Karin Tarp Wendt for invaluable help with all the qPCR-work performed in this thesis and for helpful discussions about RNA purifications (this goes for Susanna Cirera as well). Panchale Olsen and Henriette Vorsholt are thanked for assistance with the RNA purifications when I was not able to perform them myself. I also wish to thank Heidi Gertz Andersen and Margrethe Carlsen for teaching me all I know about ELISAs.

I am thankful to all the people who have taken care of the pigs at the University of Aarhus (Tjele), the University of Copenhagen (Taastrup) and the Indiana University School of Medicine and Purdue University breeding colony (West Lafayette). Without your excellent caretaking this work would not have been possible.

My deepest gratitude to my husband Morten and my wonderful son Magnus for understanding my ambitions and accepting the long hours at work. I am really looking forward to spending more time with you now! And of course my Dad for always believing in me, and for instilling the belief in me that I can do anything I set my mind to.

Last but not least, I wish to thank the rest of my family and my friends for support during these last four years. Without your help this would not have been possible.

Abstract

Overweight and obesity are increasing at an alarming rate globally, and are quickly becoming one of the largest medical problems in the world. Overweight and obesity are risk factors of metabolic diseases such as diabetes, cardiovascular diseases and cancer. Obesity is associated with a chronic state of low-grade inflammation in the adipose tissues, which involves several factors of the innate immune response having a range of systemic effects and which has been implicated in the development of the metabolic syndrome.

To investigate the impact of obesity and obesity-related diseases good translational animal models are needed, and as such pigs have been proposed as relevant models for human obesity-induced inflammation as pigs share many genetic, anatomical and physiological features with humans.

In this project the up- and downregulation of genes and proteins involved in the innate immune response to obesity-induced inflammation were examined to define the response and to identify a pig model that resembles the human response. Regulation was examined in the liver, two adipose tissues from the abdomen (subcutaneous adipose tissue (SAT) from the abdomen or retroperitoneal adipose tissue (RPAT) and visceral adipose tissue (VAT)) and one from the neck (neck SAT), as well as in the blood. Three pig breeds, all previously used as obesity models, have been examined, namely domestic pigs, Göttingen minipigs and Ossabaw minipigs, as well as cloned domestic pigs in an attempt to reduce variation, and thus the number of animals to be used in a trial to obtain statistical power.

For the gene regulation analysis, two platforms for quantitative real-time PCR (qPCR) were employed: The Rotor-Gene Q instrument and the microfluidics-based high-throughput Bio-Mark. For the serum protein concentrations analysis several enzyme-linked immunosorbent assays (ELISAs) were used on the blood.

In the clones, both cloning and obesity changed the response of the innate immune genes in the tissues and in the blood, as fewer genes were differentially regulated in the clones and in the obese, than in the controls and lean pigs. These effects were additive, so that obese, cloned pigs showed the least differential gene expression. However, the obese clones showed up-regulation of two serum proteins, namely haptoglobin and orosomucoid (ORM), as the only group of domestic pigs. Furthermore, cloning did not reduce the inter-individual phenotypic variation between the pigs. This changed expression of the innate immune response seen in cloned pigs may have phenotypic effects over time, and should therefore be taken into consideration when using cloned pigs for nutritional studies.

In the Göttingen minipigs, obesity induced extensive changes in gene expression in the tissues as nine genes in the liver (out of 35), 12 genes in the VAT, 11 in the RPAT and eight genes in the neck SAT (out of 33) were significantly differentially regulated in the obese Göttingen minipigs compared to lean. Three genes were differentially expressed in all three adipose tissues, namely the proinflammatory CC Chemokine ligand 3-like 1 (*CCL3L1)*, the anti-inflammatory *CD200* and the anti-inflammatory *IL1RN*, indicating that several factors in the adipose tissues are suppressing the effects of obesity. This response is like the response observed in obese humans. However, the obese Göttingen minipigs do not show elevated serum protein concentrations, which is observed in obese humans.

The obese Ossabaw minipigs did not show significant differential expression in the adipose tissues, although this could be an artifact due to small group sizes. However, a number of genes in the liver (six out of 26 genes) were down-regulated, which is not like the human response to obesity.

When comparing the three breeds of porcine obesity-models with regards to the acute phase protein ORM, none of the three breeds showed any difference in the gene regulation of *ORM* between the lean and obese pigs. ORM has been found to have anti-inflammatory and immunomodulatory effects and has been associated with maintaining the metabolic homeostasis in obesity. Furthermore, no expression differences were found between the adipose tissues, which is similar what has been observed in obese humans. In obese humans, raised plasma levels of ORM have been found, as was the case for obese Ossabaw minipigs. The plasma levels between lean and obese domestic pigs and Göttingen minipigs were unchanged. Therefore, with regard to ORM, the obese Ossabaw minipigs respond similarly to obesity as humans.

In conclusion, none of the investigated breeds of porcine obesity models showed a complete human-like response to obesity, under the investigated conditions, though the obese Göttingen minipigs showed a response similar to obese humans with regards to gene expression in the adipose tissues, and the obese Ossabaw minipigs showed a similar response with regard to serum ORM concentrations.

Sammendrag på dansk

Overvægt og fedme er stigende i et alarmerende tempo globalt, og er hurtigt ved at blive et af de største medicinske problemer i verden. Overvægt og fedme er risikofaktorer for metaboliske sygdomme såsom diabetes, hjerte-kar-sygdomme og kræft. Fedme er forbundet med en kronisk tilstand af let inflammation i fedtvæv, der involverer flere faktorer af det innate immunrespons med en række systemiske effekter, og som er impliceret i udviklingen af det metaboliske syndrom.

For at undersøge effekten af fedme og fedmerelaterede sygdomme er gode translationelle dyremodeller nødvendige, og som sådan er griseblevet foreslået som relevante modeller for human fedme-induceret inflammation eftersom, grise deler mange genetiske, anatomiske og fysiologiske ligheder med mennesker.

I dette projekt blev op- og nedregulering af gener og proteiner involveret i det innate immunrespons mod fedme-induceret inflammation undersøgt for at definere responset og for at identificere en grise-model, der minder om det humane respons. Reguleringen blev undersøgt i leveren, to fedtvæv fra maven (subcutant fedtvæv (SAT) fra abdomen eller retroperitoneal fedtvæv (RPAT) og visceralt fedtvæv (VAT)) samt SAT fra halsen (neck SAT), såvel som i blodet. Tre tynde og fede svineracer, som alle tidligere er anvendt som fedme-modeller, er blevet undersøgt, nemlig produktionsgrise, Göttingen minigrise og Ossabaw minigrise, samt klonede produktionsgrise i et forsøg på at reducere variation, og dermed antallet af dyr, der skal anvendes i et studie for at opnå statistisk signifikans.

I genreguleringsanalyserne blev to platforme til kvantitativ real-time PCR (qPCR) anvendt: Et Rotor-Gene Q instrument og en mikrofluid-baseret high-throughput Bio-Mark. Enzym-bundet immunsorbent-assays (ELISAs) blev anvendt for at analysere flere serumproteiners koncentrationer i blodet.

I klonerne blev både klonings- og fedmeresponset fra gener i det innate immunforsvar ændret i væv og i blodet, idet færre gener var differentielt reguleret i klonerne og i fede grise, end i kontrollerne og tynde grise. Disse virkninger var additive, således at fede, klonede grise viste den mindste differentielle genekspression. Dog viste de fede kloner opregulering af to serumproteiner, nemlig haptoglobin og orosomucoid (ORM), som den eneste gruppe af produktionsgrise. Desuden reducerede kloning ikke den inter-individuelle fænotypiske variation mellem grisene. Dette ændrede respons i det innate immunrespons kan have fænotypiske effekter over tid, og derfor bør dette tages i betragtning, når klonede grise bruges til ernærings undersøgelser.

I Göttingen minigrise inducerede fedme omfattende ændringer i genekspressionen i vævene, da ni gener i leveren (ud af 35), 12 gener i VAT, 11 i RPAT og otte gener i neck SAT (ud af 33) var signifikant differentielt reguleret i de fede Göttingen minigrise i forhold til de tynde. Tre gener blev differentielt udtrykt i alle tre fedtvæv, nemlig det proinflammatoriske CC chemokin ligand 3-like 1 (*CCL3L1*), den antiinflammatoriske *CD200* og den antiinflammatoriske *IL1RN*, hvilket indikerer, at flere faktorer i fedtvæv undertrykker konsekvenserne af fedme. Denne reaktion er lig det respons der er observeret i fede mennesker. Dog udviste de fede Göttingen minigrise ikke forhøjede serum proteinkoncentrationer, der er observeret hos fede mennesker.

De fede Ossabaw minigrise udviste ikke differentiel ekspression i fedtvævene, selv om dette kan skyldes små gruppestørrelser. En række gener i leveren (seks ud af 26 gener) var nedreguleret, hvilket ikke er lig menneskers respons på fedme.

Ved sammenligning af de tre racer af porcine fedme-modeller i forhold til akutfaseproteinet ORM, udviste alle tre racer ingen forskel i genreguleringen af ORM mellem de tynde og fede grise. ORM har vist sig at have antiinflammatoriske og immunomodulatoriske virkninger, og er blevet forbundet med at opretholde metabolisk homøostase under fedme. Endvidere blev ingen ekspressions forskelle fundet mellem fedtvævene, hvilket svarer til responset hos fede mennesker. Hos fede mennesker er forhøjede plasmaniveauer af ORM blevet fundet, hvilket også var tilfældet for fede Ossabaw minigrise. Plasmaniveauerne mellem tynde og fede produktionsgrise og Göttingen minigrise var uændrede. Hvad angår ORM, reagerer Ossabaw minigrise altså tilsvarende på fedme som mennesker.

Som konklusion viste ingen af de undersøgte racer af porcine fedme modeller et fuldstændigt menneske-lignende respons på fedme, under de undersøgte betingelser. Dog udviste de fede Göttingen minigrise et respons svarende til fede menneskers med hensyn til genekspression i fedtvæv, og de fede Ossabaw minigrise viste en lignende reaktion med hensyn til serum ORM koncentrationer i blodet.

Abbreviations

ACTB – β-actin
AOAH – acyloxyacyl hydrolase
APOA1 – Apolipoprotein A-1
APP – acute phase protein
B2M - beta-2-microglobulin
BMI – body mass index
C3 – complement component 3
CCL– chemokine (C-C motif) ligand
CCL3L1 – chemokine (C-C motif) ligand 3-like 1
cDNA – complementary DNA
COX-2 – prostaglandin-endoperoxide synthase 2
CRP – C-reactive protein
CXCL10 – chemokine C-X-C motif ligand 10
DEFB1 – defensin, β1
DNA – deoxyribonuclein acid
FIB – fibrinogen
GAPDH - glyceraldehydes-3-phosphate dehydrogenase
HP – haptoglobin
HPRT1 - hypozanthine phosphoribosyl transferase 1
IFNG – interferon γ
IL – interleukin
IL1RN – interleukin 1 receptor antagonist
ITIH4 – inter-α-trypsin inhibitor heavy chain family, member 4
LBP – lipopolysaccharide binding protein
LPS – lipopolysaccharide
MetS – metabolic syndrome
mRNA – messenger RNA
MUC1 – mucin
NFKB1 – nuclear factor of κ light polypeptide enhancer in B-cells 1
NFKBIA – nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α
NTC – no template control
ORM1 – orosomucoid 1
PAFAH1B1 – platelet-activating factor acetylhydrolase 1b
PDB-2 – defensin, β2
qPCR – quantitative real-time PCR
RIN – RNA integrity number
RNA – ribonuclein acid
RPAT – retroperitoneal adipose tissue
RPL13A - ribosomal protein L13A
-RT – no reverse transcription (control)
SAA – serum amyloid A
SAT – subcutaneous adipose tissue
SCNT – somatic cell nuclear transfer
SEM – standard error of the mean
SFTPA1 – surfactant protein A1
TF – transferring
TGFB1 – transforming growth factor β1
TLR4 – toll-like receptor 4
TNFAIP3 – tumor necrosis factor α-induced protein 3
TNF-α – tumor necrosis factor alpha
VAT – visceral adipose tissue
VEGF – vascular epithelial growth factor
WAT – white adipose tissue
WHO – World Health Organization

Content

1 Background

1.1 Obesity-induced low-grade inflammation

Overweight and obesity are increasing at an alarming rate globally (Figure 1), and are quickly becoming one of the largest medical problems in the world (Abelson & Kennedy 2004; Haslam & James 2001). In 2005, it was estimated that 937 million adults were overweight and 396 million were obese. Furthermore, obesity projections reveal that up to 2.16 billion will be overweight and as many as 1.12 billion people will be obese by 2030 (Kelly *et al.* 2008). As defined by the World Health Organization (WHO), the body mass index (BMI) is used as a tool to identify weight problems. The BMI is a person's weight in kilograms (kg) divided by their squared height in meters. Overweight is defined by a BMI=25-29.9 and obesity by a BMI≥30 (source: who.int). This is important as overweight and obesity are important risk factors of diabetes, cardiovascular disease, and cancer, and therefore increases mortality and decreases life expectancy (Haslam & James 2001).

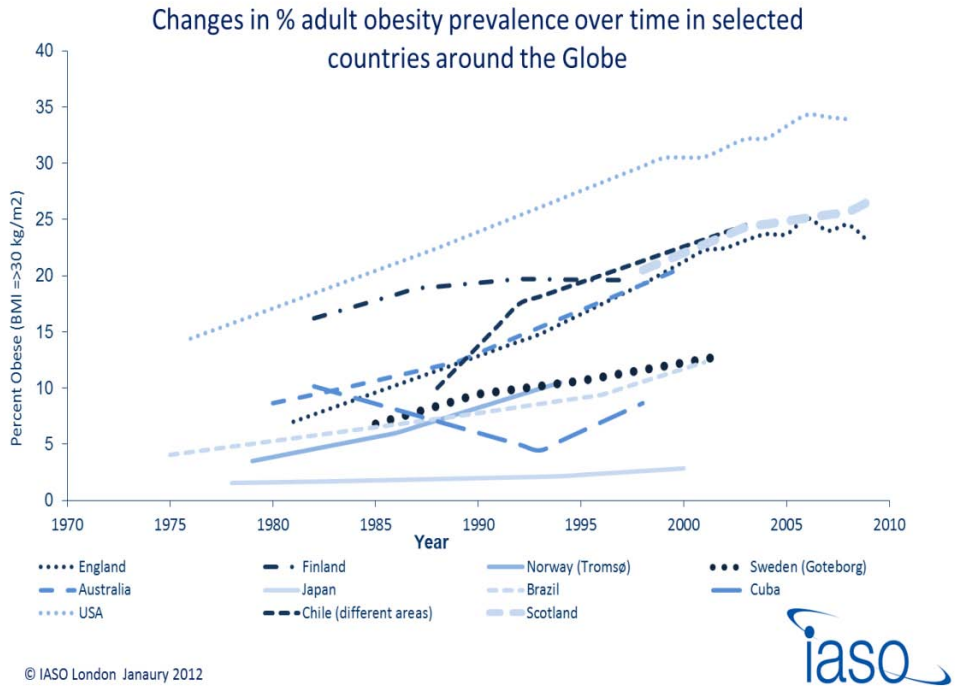

Figure 1: Overview of the changes in obesity prevalence in adults in selected countries. Source: International Association for the study of Obesity, IASO.org.

In most mammals adipose tissue occurs in two forms; the brown adipose tissue and the white adipose tissue (WAT) (reviewed in (Trayhurn & Beattie 2001)). Brown adipose tissue is found mainly in neonates and is important for the regulation of body-temperature through non-shivering thermogenesis. WAT has several functions; it is a site for energy storage of fatty acids and it also provides thermal insulation. Furthermore, WAT has endocrine functions, releasing so-called adipokines that have both local and systemic functions.

Obesity is associated with a chronic activation of the innate immune system resulting in a systemic low-grade inflammation of the WAT which has been implicated in the development of obesity-related diseases, such as insulin resistance, cardiovascular diseases and hepatic steatosis (Gil *et al.* 2007; Bastard *et al.* 2006; Federico *et al.* 2010), and the development of the metabolic syndrome (MetS) (Yudkin *et al.* 2004). The MetS is a cluster of pathologies, of which the main components include insulin resistance, atherosclerotic dyslipidaemia, elevated blood pressure, abdominal obesity, and a systemic proinflammatory state. The components of MetS differ in criteria defined by different organizations (for example WHO and ATP III),

however, the before mentioned are generally agreed upon (Grundy *et al.* 2004). At least 3 of these risk factors need to be present for a diagnosis of MetS to be made.

The obesity-induced low-grade inflammation is different from "normal" acute inflammation caused by pathogens, where inflammation is a principal response to injuries of the body, with hallmarks that include swelling, redness, pain and heat. The acute inflammation response is rapid, robust and transient, with important mechanisms for the regulation and resolution of the inflammation (Serhan & Savill 2005; Zhang & Spite 2012; Mannick *et al.* 2001). Acute inflammation involves integration of many complex cellular and soluble signals in distinct cells and organs locally and systemically (Gabay & Kushner 1999; Tilg & Moschen 2006). Most of the cytokines and acute phase proteins (APPs) involved in a normal inflammatory response are produced mainly in the liver. In contrast, obesity-induced low-grade inflammation is long-term with often only small changes in the pattern of the immune factors involved (Fain 2010) and several proteins involved in this process are synthesized and secreted by the adipose tissues as well, such as C-reactive protein (CRP), interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) (reviewed in (Fain 2010; Fantuzzi 2005)). Therefore, the low-grade inflammation of the adipose tissues is not only local, but also has systemic implications. It is still unknown whether low-grade obesity-induced inflammation is caused by defects in the program to resolve inflammation or whether it is induced by a continuous attenuated stimulation of inflammation (Zhang & Spite 2012).

Even though obesity can induce low-grade inflammation, it is not the adiposity in itself, but rather the body fat distribution that plays a role in the development of metabolic diseases. Abdominal visceral adipose tissue (VAT) is more closely associated with an adverse metabolic risk profile, including cardiovascular disease, than subcutaneous adipose tissue (SAT) (Fox *et al.* 2007) and thigh SAT may even exhibit beneficial effects on glucose and lipid levels independent of abdominal adipose tissue (Snijder *et al.* 2005), whereas abdominal SAT seems to have a beneficial effect on triglyceride levels in obese humans (Porter *et al.* 2009). There are two main hypotheses currently, not mutually exclusive, as to why VAT is more involved in the low-grade inflammation than the SAT (Federico *et al.* 2010; Klein *et al.* 2007). The first hypothesis proposes a direct effect of VAT on insulin resistance, lipoprotein metabolism and blood pressure through secreted metabolic products such as free fatty acids. The VAT is drained by the portal vein, which provides direct delivery of metabolic products of VAT to the liver, and it has been shown that secretion from the VAT of adipokines is associated with systemic inflammation in obese humans (Fontana *et al.* 2007). The second hypothesis, called the ectopic hypothesis, suggests that SAT depots have a limited ability to store excess energy, which ultimately leads to overflow of fatty acids to intra-abdominal adipose tissue and ectopic sites such as liver, muscle and islets. The excessive ectopic fat then causes metabolic dysfunction in those organs, such as insulin resistance and dyslipidemia (Seppälä-Lindroos *et al.* 2002; Sinha *et al.* 2002), and a correlation between macrophage activation and plasma levels of free fatty acids has been found (Ghanim *et al.* 2004).

The exact cause(-s) of obesity-induced low-grade inflammation is not known, but there are many theories. When exposed to over-nutrition with consequent expansion of adipose tissue (including increase in adipocyte size and numbers, as well as adipose tissue proliferation), a number of local physiological changes occur in the adipose tissue; alterations in fatty acid metabolism (Suganami *et al.* 2005; Shi *et al.* 2006), vascularisation (Hosogai *et al.* 2007; Pang *et al.* 2008), local hypoxia (Hosogai *et al.* 2007; Ye *et al.*

2007), adipocyte cell death (Strissel *et al.* 2007), and adipocytes secretion (Skurk *et al.* 2007; Clément *et al.* 2004). These changes are hypothesized to be important for the development of low-grade inflammation.

The main cell type in adipose tissue is adipocytes, but adipose tissue also consists of preadipocytes, vascular endothelial cells, fibroblasts, and immune cells such as lymphocytes and macrophages (Ouchi *et al.* 2011). Recently, there has been great focus on the recruitment of macrophages in the adipose tissues as contributors to inflammatory and metabolic alterations in obesity (for example reviewed in (Dalmas *et al.* 2011; Lumeng 2010)), as the infiltration of macrophages is directly linked to systemic inflammation and insulin resistance and is also proportional to adiposity in humans and mice (Weisberg *et al.* 2003; Xu *et al.* 2003). The percentage of macrophages in adipose tissue of lean humans is estimated to be around 10% and around 40% in obese humans (Weisberg *et al.* 2003). Furthermore, the number of macrophages in VAT is greater than in the SAT (Bruun *et al.* 2005) supporting the fact that VAT is more involved in the low-grade inflammation than SAT. However, it is still unclear exactly what triggers the infiltration or how it occurs. It is still debated how much the infiltrating macrophages contribute to the secretion of adipokines in the WAT in obesity, however, there appears to be some overlap in the secretion from macrophages and adipocytes, as several of the proteins released by the fat cells are released by the nonfat cells in the adipose tissues as well (Fain 2010).

1.1.1 Adipose tissue as an endocrine organ

Nowadays, the term adipokine covers any protein that can be synthesized and secreted by adipose tissue (Trayhurn & Wood 2004). The adipokines released from immune cells and adipocytes make up a complex network that orchestrates interactions between metabolism and the immune system (Figure 2). Elevated serum concentrations and adipose tissue expression of these adipokines have been found in obese individuals (Table 1). Furthermore, several of the proteins shown in Table 1 have been shown to have an effect on the development of metabolic diseases in obesity, and on the predictability of weight gain (Engström *et al.* 2003). There are several adipokines that are secreted exclusively by the adipose tissues and that exerts great effects on the state of inflammation, such as leptin, resistin, visfatin and adiponectin. Adiponectin is the only adipokine from this group that will be discussed further in this thesis, as the main focus will be on innate immune factors. As mentioned and shown in Table 1, the secreted adipokines and their effects on metabolic disorders are complex. For this reason only a few adipokines will be highlighted in the following sections.

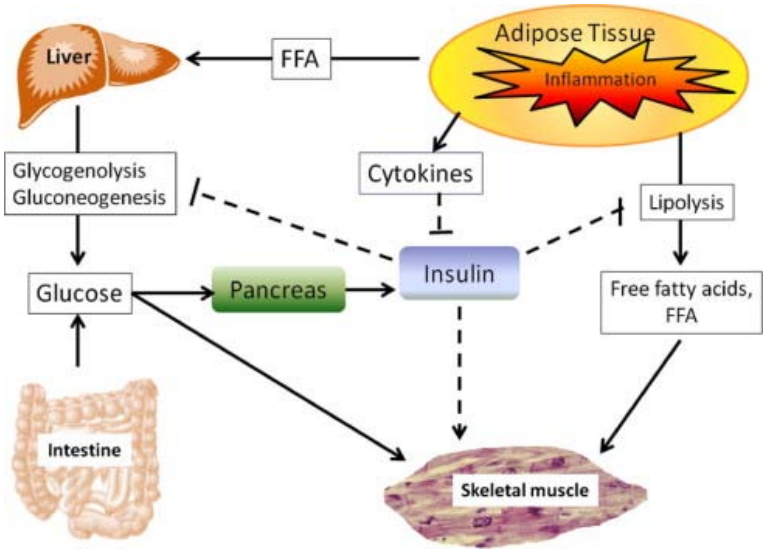

Figure 2: Local and systemic effects of low-grade inflammation of adipose tissue. Source: (Kalupahana *et al.* 2012)

Some of the best studied adipokines in obesity are IL-6 and TNF-α and they have consistently been found to be increased by obesity in the serum, WAT or both (see Table 1, reviewed in (Cottam *et al.* 2004)). The adipose tissue secretes about 30% of the circulating IL-6 in obese individuals (Mohamed-Ali *et al.* 1997) and both VAT and SAT secretes IL-6 (Fontana *et al.* 2007; Mohamed-Ali *et al.* 1997). The direct secretion of IL-6 into the portal vein has important metabolic consequences as IL-6 exerts a lot of hepatic effects; it stimulates hepatic APP production (Heinrich *et al.* 1990), including CRP (Fontana *et al.* 2007), impairs insulin-mediated glycogenesis (Senn *et al.* 2002) and stimulates hepatic gluconeogenesis (Tsigos *et al.* 1997). Furthermore, increased IL-6 serum levels are associated with increased risk of type 2 diabetes (with CRP) and cardiovascular diseases (Pickup *et al.* 1997; Pradhan AD 2001; Plutzky 2001). TNF-α has many functions in obese individuals; it correlates with the level of dyslipidemia (Chan *et al.* 2002), inhibit lipoprotein lipase (Kern *et al.* 1995) and increase lipolysis (Grunfeld *et al.* 1989). However, even though the expression of TNF-α is upregulated in the adipose tissue of obese individuals compared to lean (Hotamisligil *et al.* 1995) and the serum levels is higher in obese individuals (Dandona *et al.* 1998), it has been shown that there is no net release of TNF-α from abdominal SAT (Mohamed-Ali *et al.* 1997). The plasma concentrations of TNF-α were similar in the portal vein as in the radial vein, indicating that the upregulated expression of TNF-α in the adipose tissue does not have a systemic effect, but rather a local as TNF-α is not primarily released into the bloodstream from the adipose tissues (Fontana *et al.* 2007). It can be hypothesized that the elevation in TNF-α serum levels leading to metabolic disorders is due to secretion from other tissues. TNF-α has been implicated in the development of insulin resistance (Hotamisligil *et al.* 1995) as TNF-α can inhibit intracellular signaling from the insulin receptor (Hotamisligil *et al.* 1994) and is positively associated with cardiovascular risk factors (Mendall *et al.* 1997).

Several APPs have been investigated as possible markers of the disturbance of metabolic homeostasis during obesity, either secreted from the adipose tissues or secreted from other tissues due to secreted adipokines. CRP is mainly a hepatically produced APP, but it is also secreted from adipose tissues (Fain 2010). The serum levels of CRP are elevated during obesity (Khaodhiar *et al.* 2004; Esposito *et al.* 2002; van Dielen *et al.* 2001), and IL-6 is one of the main inducers of hepatic CRP production in most species (Weinhold & Rüther 1997) which involves CRP in many of the metabolic disorders IL-6 is involved with. Furthermore, CRP has been proposed as a marker of cardiovascular disease. Studies have shown that high-normal base-line CRP levels are associated with increased risk of coronary events, even in healthy, normal-weight individuals, with lipid levels below the median levels of the population (Ridker *et al.* 1998; Ridker *et al.* 2000).

Serum amyloid A (SAA) is another APP reported to be increased in serum and adipose tissue of obese humans (Poitou *et al.* 2005; van Dielen *et al.* 2001). Chronic systemic elevation of SAA has been linked to metabolic diseases and it is a well-established risk factor for atherosclerosis (Ridker *et al.* 2000; Fyfe *et al.* 1997; Liuzzo *et al.* 1994). The correlation of gene expression with plasma levels was higher in adipose tissue than in the liver, and even though the liver is believed to be the most important organ for SAA secretion in the acute phase response, insulin-related human studies have shown that *SAA* expression is higher in adipose tissues than in the liver (Poitou *et al.* 2006). SAA is induced in primarily by IL-1, TNF-α and IL-6 in most species (Gabay & Kushner 1999).

Adiponectin is highly expressed in adipose tissue and adiponectin plasma levels constitutes 0.01% of circulating proteins, making adiponectin one of the most abundant adipokines in plasma (Bastard *et al.*

2006). However, the expression of adiponectin mRNA is dependent on the localization of adipose tissue as the expression is higher in abdominal SAT than in VAT (Lihn *et al.* 2004). Adiponectin differs from other adipokines as the circulating levels are decreased in obese humans and in patients with type 2 diabetes and coronary heart disease (Kern *et al.* 2003; Spranger *et al.* 2003; Weyer *et al.* 2001; Ouchi *et al.* 1999). Furthermore, there is a strong positive correlation between decreased adiponectin and insulin sensitivity (Weyer *et al.* 2001). As adiponectin has anti-inflammatory effects, it has been suggested that the decreased levels of adiponectin in obesity and metabolic diseases are a contributing factor to the low-grade inflammation, and that adiponectin has a protective role against insulin resistance and atherosclerosis. One of these anti-inflammatory effects is prevention of the formation of so-called foam cells; lipid-repleted macrophages, which are a feature of atherosclerosis, and which produce high amounts of proinflammatory cytokines (Tian *et al.* 2012). Another anti-inflammatory effect is the inhibition of expression of different vascular adhesion molecules, scavenger receptors and proinflammatory cytokines, such as TNF-α, IL-6 and IL-1β in various tissues (Ajuwon & Spurlock 2005; Tsatsanis *et al.* 2005). Interestingly, adiponectin has also been implicated in chronic inflammatory processes, as the expression of adiponectin is elevated in inflamed tissues and circulating levels of adiponectin are higher in patients with chronic inflammatory diseases, such as inflammatory bowel disease, osteroarthritis and rheumatoid arthritis (Tang *et al.* 2007; Ehling *et al.* 2006; Fayad *et al.* 2007). Adiponectin has been suggested as a marker for MetS, as plasma levels of adiponectin was negatively correlated with many parameters of the MetS, including BMI, triglycerides, cholesterol and plasma glucose and insulin (Ryo *et al.* 2004).

IL-10 is an interesting adipokine as it seemingly has dual roles. IL-10 is a well-known anti-inflammatory protein, and its functions include inhibition of the cytokine synthesis, suppression of macrophage function and inhibition of proinflammatory cytokines (Tedgui & Mallat 2001). Furthermore, IL-10 has been shown to block the formation and stability of atherosclerotic lesions (Pinderski Oslund *et al.* 1999). However, IL-10 also has proinflammatory functions, as discussed in a review by Mocellin and coworkers, as IL-10 promotes the activation of NK-cells, stimulates T cells and enhances the expression of cytotoxic molecules (Mocellin *et al.* 2003). Furthermore, when administered to humans with inflammatory disorders intravenously, IL-10 exhibits proinflammatory effects through release of IFN-γ, among others (Lauw *et al.* 2000). The circulating levels of IL-10 are elevated in obese humans compared to non-obese humans (Esposito *et al.* 2003; Juge-Aubry *et al.* 2005), with a 2 fold-increase in the SAT and a 6-fold increase in the VAT of obese humans when compared to the SAT and VAT of lean humans (Juge-Aubry *et al.* 2005). However, low IL-10 levels are detected in both obese and non-obese humans with MetS (Esposito *et al.* 2003). Low serum levels of IL-10 have been associated with increased risks of stroke and type 2 diabetes in humans (van Exel *et al.* 2002a; van Exel *et al.* 2002b). The higher IL-10 levels in obese individuals could be part of a response to inhibit the proinflammatory cytokine and chemokine production observed in obesity.

Gene symbol	Gene name	Change in expression levels in obesity	Family	Function	Reference
ADIPOQ	Adiponectin	↓	Adipokine	Modulates several metabolic processes	(Kern *et al.* 2003; Diez & Iglesias 2003)
AOAH	Acyloxyacyl hydrolase	N/A	Immune regulatory	Phospholipase activity	(Munford & Hunter 1992)
APOA1	Apolipoprotein A-1	↑	APP	Stimulates the innate immune response and couples cholesterol trafficking to inflammation	(Smoak *et al.* 2010; Chu *et al.* 2001)
C3	Complement component 3	↑	APP	Part of the complement system	(Gabrielsson *et al.* 2003)
CCL2	Chemokine (C-C motif) ligand 2	↑	Chemokine	Macrophage recruitment into adipose tissues	(Christiansen *et al.* 2004; Kanda *et al.* 2006)
CCL3L1	Chemokine (C-C motif) ligand 3-like 1	↑ (in mice)	Chemokine	Involved in recruitment and activation of polymorphonuclear leukocytes	(Wolpe *et al.* 1989; Xu *et al.* 2003)
CCL5	Chemokine (C-C motif) ligand 5	↑	Chemokine	Recruitment and anti-apoptotic activity of macrophages	(Keophiphath *et al.* 2010)
CD14	CD14	↑	Immune regulatory	Cell surface protein required for the immune response to LPS	(LeVan *et al.* 2008; Fain *et al.* 2010b)
CD163	CD163	↑	Immune regulatory	Plays a role in the anti-inflammatory response	(Zeyda *et al.* 2007; Moestrup & Møller 2004)
CD200	CD200	↑	Immune regulatory	Suppresses activation of macrophages and granulocytes via CD200R	(Zeyda *et al.* 2007; Hoek *et al.* 2000)
CD36	CD36	↑	Immune regulatory	Involved in fatty acids transport	(Bonen *et al.* 2006; Ibrahimi & Abumrad 2002)
CD40	CD40	↑	Immune regulatory	Triggers inflammatory response in adipocytes	(Poggi *et al.* 2009)
COX-2	Prostaglandin-endoperoxide synthase 2	↑	Immune regulatory	The key enzyme in eicosanoid metabolism	(Bolduc *et al.* 2004; Hsieh *et al.* 2009)
CRP	C-reactive protein	↑	APP	Marker of inflammation	(Esposito *et al.* 2003; Khaodhiar *et al.* 2004; van Dielen *et al.* 2001; Couillard *et al.* 2005; Festa *et al.* 2001)
CXCL10	Chemokine C-X-C motif ligand 10	↑	Chemokine	Chemoattractant, induced by IFN-γ	(Herder *et al.* 2006)
DEFB1	Defensin, β1	N/A	Immune regulatory	Anti-microbiel peptide, involved in asthma	(Tesse *et al.* 2011)
FIB	Fibrinogen	↑	APP	Marker of inflammation	(Festa *et al.* 2001; Maresca *et al.* 1999)
HP	Haptoglobin	↑	APP	Marker of inflammation	(Chiellini *et al.* 2004)
IFNG	Interferon γ	↑ (in mice)	Cytokine	Immune-stimulatory and –modulatory effects	(Guebre-Xabier *et al.* 2000; Schoenborn & Wilson 2007)
IL10	Interleukin 10	↑ (↓ in MetS)	Cytokine	Anti- and proinflammatory	(Esposito *et al.* 2003; Mocellin *et al.* 2003)
IL12A	Interleukin 12A	↑	Cytokine	Proinflammatory	(Silswal *et al.* 2005; Yang *et al.* 1997)
IL18	Interleukin 18	↑	Cytokine	Proinflammatory	(Simon *et al.* 2009; Esposito *et al.* 2002)

IL1B	Interleukin 1, β	↑	Cytokine	Several; for example stimulate lipolysis, glucose transport, and adipocyte maturation in adipose tissue	(Maury *et al.* 2007; Arkan *et al.* 2005; Juge-Aubry *et al.* 2003)
IL1RN	Interleukin 1 receptor antagonist	↑	Cytokine	Counteract inflammatory effects of IL-1 family members	(Juge-Aubry *et al.* 2003; Sauter *et al.* 2008)
IL6	Interleukin 6	↑	Cytokine	Anti- and proinflammatory	(Esposito *et al.* 2003; Khaodhiar *et al.* 2004; Hirano *et al.* 1990)
IL8	Interleukin 8	↑	Chemokine	Secreted by macrophages, is a chemoattractant of neutrophils	(Straczkowski *et al.* 2002; Kim *et al.* 2006)
ITIH4	Inter-α-trypsin inhibitor heavy chain family, member 4	↑ (in rats)	APP	Marker of inflammation	(Choi *et al.* 2010)
LBP	Lipopolysaccharide binding protein	↑	APP	Recognizes LPS and amplifies host response to LPS	(Sun *et al.* 2010; Weiss 2003; van Dielen *et al.* 2001)
MUC1	Mucin	N/A	Immune regulatory	Binds pathogens so they cannot reach the cell surface	(Moncada *et al.* 2003)
NFKB1	Nuclear factor of kappa light polypeptide enhancer in B-cells 1	↑	Immune regulatory	Transcription factor in the inflammatory cascade	(O'Hara *et al.* 2009; Fain *et al.* 2010a; Creely *et al.* 2007; Ghanim *et al.* 2004)
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, α	↓	Immune regulatory	Inhibits transcription factor NF-κB	(O'Hara *et al.* 2009; Ghanim *et al.* 2004)
ORM1	Orosomucoid 1	↑	APP	Protection of adipose tissue against inflammation and metabolic dysfunction	(Lee *et al.* 2010; Alfadda *et al.* 2012; van Dielen *et al.* 2001)
PAFAH1B1	Platelet-activating factor acetylhydrolase 1b	↑ (in mice)	Immune regulatory	Hydrolyses and inactivates platelet activating factor (PAF)	(Yamada & Yokota 1998; Becerril *et al.* 2012)
PDB-2	Defensin, β2	N/A	Immune regulatory	Various immune functions	(Liu *et al.* 1998)
SFTPA1	Surfactant protein A1	N/A	APP	Various inflammatory functions	(van de Wetering *et al.* 2004)
SAA	Serum amyloid A	↑	APP	Marker of inflammation	(Poitou *et al.* 2005; van Dielen *et al.* 2001)
TF	Transferrin	↔	APP	Binds iron	(POMEROY *et al.* 1997)
TGFB1	Transforming growth factor β 1	↑	Cytokine	Pro- and anti-inflammatory functions	(Scaglione *et al.* 2003; Letterio & Roberts 1998)
TLR4	Toll-like receptor 4	↑	Immune regulatory	Proinflammatory, activated by LPS and saturated fatty acids	(Shi *et al.* 2006; Lee *et al.* 2003; Tsukumo *et al.* 2007)
TNF	Tumor necrosis factor-α	↑	Cytokine	Proinflammatory, insulin inhibitory actions, regulates immune cells	(Cartier *et al.* 2008; Khaodhiar *et al.* 2004; Dandona *et al.* 1998)
TNFAIP3	Tumor necrosis factor α-induced protein 3	↓	Cytokine	Negative feedback inhibitor of transcription factor NF-κB	(Amar *et al.* 2007; Boonyasrisawat *et al.* 2007)

Table 1: List of investigated genes, including functions and changes in expression levels in obesity. N/A means no data is available. APP means acute phase protein. MetS means the metabolic syndrome. LPS means lipopolysaccharide.

1.1.2 The cause of low-grade inflammation

As previously described, the causes of obesity-induced low-grade inflammation are not known, but a few theories will be discussed in this section.

1.1.2.1 Hypoxia-induced low-grade inflammation in obesity

Hypoxia is a known initiator of inflammation (Eltzschig & Carmeliet 2011) and has been proposed as a cause of obesity-induced low-grade inflammation. This is due to the facts that the oxygen tension in obese patients was reduced in the subcutaneous tissue (Fleischmann *et al.* 2005; Kabon *et al.* 2004), that mRNA of hypoxia response genes were increased in adipose tissue of the *ob/ob* mice (Ye *et al.* 2007), that hypoxia stimulates secretion of leptin and vascular epithelial growth factor (VEGF) and hypoxia inhibits differentiation of preadipocytes into mature adipocytes *in vitro* (Hosogai *et al.* 2007; Ye *et al.* 2007; Yun *et al.* 2002; Lolméde *et al.* 2003; Chen *et al.* 2006; Wang *et al.* 2007). Furthermore, hypoxia can induce gene expression in adipocytes and macrophages, such as *TNF-α*, *IL-6* and *IL-1*, and thereby induce inflammation in the adipose tissues (Ye *et al.* 2007; Hosogai *et al.* 2007) and it is known to inhibit macrophage departure from hypoxic regions in tissues (Turner *et al.* 1999).The induction of hypoxia in adipose tissues may be due to the alterations in vascularisation caused by obesity; obesity leads to a reduction in vascular density in *ob/ob* mice (Pang *et al.* 2008) and a reduction of adipose tissue blood flow of 30-40% in obese humans (Bolinder *et al.* 2000). In addition, the increase in adipocytes size in obesity may in itself be a contributor of hypoxia and cause problems with oxygen supply in the adipose tissues, as the diameter of a large adipocyte can be over 150 µm (Blüher *et al.* 2002) and oxygen is only able to diffuse about 120 µm (Helmlinger *et al.* 1997; Torres Filho *et al.* 1994). Therefore, as adipocyte diameter increases as obesity progresses, oxygen will not be able to reach the cells beyond 120 µm from the capillary. Furthermore, hypoxia could be the cause of the decrease of adiponectin in low-grade inflammation described previously, as hypoxia has been shown to reduce the expression of adiponectin in adipocytes in obesity (Ye *et al.* 2007; Hosogai *et al.* 2007; Chen *et al.* 2006; Wang *et al.* 2007). This decrease could possibly be through the induction of TNF-α by hypoxia which inhibits adiponectin mRNA expression in adipocytes (Fasshauer *et al.* 2002). As adiponectin has anti-inflammatory effects, as previously described, the decrease in adiponectin caused by hypoxia could exacerbate the hypoxia-induced inflammation in obesity.

Hypoxia has been proposed as a cause of adipocyte cell death in adipose tissue (Yin *et al.* 2009), which induces macrophage infiltration (Cinti *et al.* 2005). In obese individuals, especially those displaying metabolic dysfunctions, macrophages are found in aggregates or in multinucleate giant cells. These cells are located in relation to, and surrounding, single dead or moribund adipocytes in socalled crown-like structures (Weisberg *et al.* 2003; Cinti *et al.* 2005) and the macrophages in these crown-like structures have higher expressions of TNF-α and IL-6 in obese mice (Strissel *et al.* 2007). Furthermore, macrophages in the adipose tissue of obese mice were found mainly in hypoxic areas (Rausch *et al.* 2007). As hypothesized by Ye (Ye 2008) the infiltration of macrophages in adipose tissue can be to remove the dead adipocytes from the tissues, or it can be that the macrophages are simply trapped in the hypoxic area, as previously described.

1.1.2.2 Gut bacteriel LPS-induced low-grade inflammation in obesity

Generally, there is consensus about the concept that obesity is influenced by lifestyle factors and host susceptibility, and that obesity is caused by a dysbalance between energy intake and expenditure. As an organism, humans consist of 10% human cells and 90% microbes (Savage 1977), and several host-microbe interactions affecting human health has been described, including obesity (for example reviewed in (Mai & Draganov 2009)). Studies performed in mice lacking gut microbiota (germ-free) show that microbiota are involved in the extraction of calories and the storage of these in adipose tissues as the germ-free mice contained 40% less body fat than mice with normal gut microbiota, even though the food intake of the germ-free mice were 30% higher than the mice with normal gut microbiota (Bäckhed *et al.* 2004). This lower percentage of body fat in the germ-free mice was normalized within 2 weeks when the germ-free mice were colonized with bacteria from mice with normal gut microbiota (Bäckhed *et al.* 2004), and the body fat mass gain was even more pronounced (20% higher) when colonized with bacteria from ob/ob mice (Turnbaugh *et al.* 2006). These differences is likely due to the fact that the composition of microbiota are different in lean compared to obese mice and humans with a fall in gram-negative bacteria and a rise in gram-positive bacteria (Ley *et al.* 2006; Ley *et al.* 2005). However, a group of gram-positive bacteria (bifidobacteria) was also reduced in high-fat diet-fed mice (Cani *et al.* 2007), and these have been shown to reduce lipopolysaccharide (LPS) intestinal levels in mice and improve mucosal bacterial function in rats (Griffiths *et al.* 2004; Wang *et al.* 2006). LPS is a large molecule that consists of lipid and polysaccharide fractions, and it is a strong trigger of inflammation as it initiates secretion of proinflammatory cytokines of innate immune cells, when it binds to CD14 and TLR4 on the surface of these cells (Sweet & Hume 1996; Wright *et al.* 1990). LPS has been proposed to initiate and maintain a low-grade inflammation state when feeding on a high-fat diet. A high-fat diet induced higher levels of LPS in the plasma of mice and humans, and when LPS was diluted in oil the ingestion of LPS was acute and led to elevated plasma levels of LPS (Cani *et al.* 2007). Endogenous LPS is continually produced within the gut by the death of gram-negative bacteria and is translocated into intestinal capillaries through a mechanism involving TLR4 (Neal *et al.* 2006). Furthermore, an increased concentration of fatty acids has been shown to impair the integrity of the intestinal barrier, and thereby increase the permeability of the gut (Brun *et al.* 2007; Velasquez *et al.* 1993). Coupled with the fact that the levels of CD14 and TLR4 are elevated during obesity (Table 1) this could be two possible explanations of the increase in plasma LPS levels in obesity.

LPS has been implicated in the development of metabolic diseases related to obesity. To evaluate the effect of mildly increased plasma LPS levels on insulin resistance, Cani and coworkers infused mice continually with LPS over a period of 4 weeks and found that mice infused with LPS had increased hepatic insulin resistance and heavier liver weight than compared to normal mice (Cani *et al.* 2007). This has been confirmed in human studies as LPS has been found to be involved in the development of type 2 diabetes in humans as well (Creely *et al.* 2007). Furthermore, a relationship between the plasma levels of LPS and the development of atherosclerosis and cardiovascular disease in humans have been found (Wiedermann *et al.* 1999).

1.2 Animal models of obesity

There are several good cellular model systems to study the regulation of adipocytes, including the 3T3-L1, an adipocyte differentiated from a fibroblast. It is however, not possible for studies of more complex biological systems to be performed in cell cultures. Therefore, we turn to animal models. There are many good rodent animal models for obesity, including the *ob/ob* mice and the Zucker rat, both of which have leptin receptor mutations. Furthermore, most rodents can become obese on high-fat diets, called diet-induced obesity (DIO) rodent models. However, the metabolic parameters in these rodent models are largely dependent on the strain, gender and source of dietary fat (Gajda *et al.* 2007). In the early days of research into the complexities of and correlation between obesity and MetS, rodents were widely used, and several adipokines were discovered in rodent models. However, as the same adipokines were investigated in humans the results often differed markedly. As discussed in the commentary by Arner (Arner 2005) the rodent models differ substantially in the roles of adipokines IL-6, TNF-α, resistin and adipsin, and adiponectin levels in Zucker rats are elevated in obesity (Oana *et al.* 2005) in contrast to the decreased levels in obese humans, as described previously. Furthermore, there are other metabolic and physiological differences between rodents and humans, as discussed in (Varga *et al.* 2010). Therefore, new and more compatible animal models are needed to enable the translation of biomedical research findings in animal models into relevant therapies for obesity and its comorbidities in humans.

		Swine		
	Humans	**Domestic**	**Minipig**	**Rats**
Adult BW (kg)	70	60-280	20-50	0.25-0.4
Heart size-to-BW ratio	-	Similar[1]		Not similiar[1]
Pooling of tissues (e.g. adipose)	Not required	Not required		Required
Omnivore	Yes	Yes		Yes
Tooth structure	-	Similar[1]		Not similiar[1]
Middle ear	-	Similar[1]		Not similiar[1]
Skin	-	Similar[1]		Similar[1]
Atherosclerosis	-	Similar[1]		Not similiar[1]
Kidney structure and function	-	Similar[1]		Not similiar[1]

Table 2: Comparison of general anatomical/physiological factors between humans, swine and rats. [1]Similarity compared to humans. BW means bodyweight. Reproduced from (Litten-Brown *et al.* 2010).

One of these animal models is the pig. Pigs are becoming increasingly popular as translational models for human disease as they share many genetic, anatomical and physiological similarities to humans ((Lunney 2007; Litten-Brown *et al.* 2010) and Table 2). There are several advantages with working with pigs as they easily accept a human diet, and have large adipose tissue depots and organs so it is not necessary to pool samples to gain large enough samples for further analysis (Spurlock & Gabler 2008), it is possible to use standard medical technologies to image internal organs and vessels and it is possible to collect blood samples repeatedly (Lunney 2007). Furthermore, pigs share a high sequence and chromosome structures with humans (Lunney 2007) and it is possible to reduce the genetic variation in pigs markedly by using pigs from the same litter, or cloned or transgenic pigs (Litten-Brown *et al.* 2010). One of the drawbacks of using pigs instead of rodents is pricing. Pigs are more costly to feed, house and medicate and minipigs especially, can be in short supply and expensive to purchase. Another drawback is ethical considerations as the pig is a large experimental animal.

The pig has been investigated thoroughly as a model for nutrition (Miller & Ullrey 1987), and it has been used for research on metabolic disorders (Lunney 2007), including atherosclerosis (Turk & Laughlin 2004; Turk *et al.* 2005). In regards to MetS, the pig expresses two different genotypes; a lean and an obese. Generally, domestic pigs have a long history of selective breeding for lean meat, and fall under the lean genotype category. However, the domestic pig display patterns of atherosclerotic alterations when fed excessively that are similar to those observed in obese humans (Brambilla & Contafora 2004). This makes the domestic pig an comparable model for atherosclerosis, however, it has been difficult to induce type 2 diabetes in these pigs, except when administering streptozotocin (a β-cell eradicating drug) (Spurlock & Gabler 2008) (described further in Section 3.1.2). Different breeds of minipigs are of the obese genotype. For more extensive studies of obesity and cardiovascular diseases, the minipig breeds have proven useful, and some breeds, namely the Ossabaw and Göttingen minipigs, show symptoms of insulin resistance when fed excessively (Dyson *et al.* 2006; Johansen *et al.* 2001). This is further described in Section 4.1.1.

Investigations into IL-6, TNF-α and leptin has shown that their expression in plasma and adipose tissue is elevated by obesity in pigs (Gondret *et al.* 2012; Jacobi *et al.* 2004), and that the expression of adiponectin is decreased with adiposity in pigs (Jacobi *et al.* 2004). These changes correlates to what has been found in obese humans, as described previously. All these factors taken together prove that the pig, with Ossabaw and Göttingen minipigs in particular, may play an important role in defining the molecular basis of obesity and its comorbidities.

2 General methods

This section contains a brief description into the background of the methods used in this thesis as the methods and materials are described in detail in the included papers and appendices.

2.1 Gene expression

DNA (deoxyribonucleic acid) is the genetic material of the cells, and it codes for proteins indirectly via RNA (ribonucleic acid) through the processes of transcription and translation. RNA is single-stranded, and is very similar to the copied DNA, except for the base uracil instead of thymine in DNA. There are different forms of RNA which have different functions in the cells. Messenger RNA (mRNA) is generated as a primary complementary transcript of a DNA sequence coding for a specific gene (transcription). The mRNA is exported to the ribosome in the cytoplasm, where the information of the mRNA is translated into a protein. Many events can impact this process, including enzymes that break down mRNA, thereby regulating the expression patterns of the cell. mRNA is rapidly turned over and is degraded and rendered nonfunctional at some point in the process of protein translation. This means that protein expression and regulation is very sensitive to changes in the rate of mRNA synthesis (transcriptional control). Furthermore, the RNA molecule is more unstable than the DNA molecule, because of the single-stranded structure. The stability of mRNA may be controlled by adenylation or by binding of the regulatory microRNA, that is associated with repression of transcriptional activity (Li 2002; Morgan *et al.* 2005) (post-transcriptional control). In addition there are several mechanisms to control protein production and location at the post-translational level, for example through regulating the stability of the protein by glycosylation by proteases and through signal peptides destining the protein for export, membrane or cytosol location.

These things taken together make purification of RNA from different tissues a challenging process, and the quality of the RNA purified can have great impact on the results of the investigation.

2.1.1 Purification of RNA

To achieve the highest quality possible of RNA and the highest yields from samples, it is important that the preservation and storage of these samples minimize degradation. The highest and most pure RNA yields are acquired by dicing the freshly sampled tissue into smaller pieces followed by rapid submersion in liquid nitrogen (Bustin & Nolan 2004). After preservation the samples can be safely stored at -80°C until use, as such low temperatures prevent activity of RNA degrading enzymes called RNases. The degradation of RNA by these enzymes is one of the main factors contributing to biases and inconsistencies when estimating gene expression. Furthermore, just as the time between tissue sampling and preservation should be kept at a minimum, it is important that the samples do not thaw prior to sample homogenization with an RNase inhibiting agent (see below).

There are several methods to purify RNA from a tissue sample. When choosing a method, many considerations have to be made as the choice of method can interfere with the recovery, abundance and the composition of various transcripts of RNA in the sample. Furthermore, different tissues contain different concentrations of RNA and the composition of the sample in itself, as well as the origin of the sample, may interfere with the extraction of RNA (as described below in section 2.1.1.1). Ideally, when RNA has been extracted from the sample, it should be free of contaminants such as genomic DNA, proteins,

Mg^{2+} and phenol, it should be undegraded, and it should be free of enzyme inhibitors (Fleige & Pfaffl 2006). All these contaminants can have great impact on the downstream procedures.

In order to assure that these factors are addressed, it is important to access the quantity and quality of the extracted RNA. To assess the quantity of a sample, a NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies Inc, USA) was used to estimate the concentration of nucleic acids. Aside from measuring the quantity of RNA in the sample, the NanoDrop can be used to measure contamination of a sample. The spectrophotometer utilizes the fact that different compounds absorb ultraviolet light in specific patterns: Proteins absorb light at 260 nm, nucleic acids at 260 nm, phenol at 270 nm and organic compounds at 230 nm. To estimate the contamination of proteins and phenol in a nucleic acid sample the ratio of absorbance at 260 and 280 nm ($A_{260/280}$) is used. Especially contamination of phenol has an impact on this ratio. To estimate the contamination of organic compounds in a nucleic acid sample the ratio of absorbance at 260 and 230 nm ($A_{260/230}$) is used. Both ratios should ideally be about 2.0 for RNA, which indicates no contamination, but ratios between 1.8 and 2 are generally accepted. However, as demonstrated by the results of the work done in this thesis, it appears that even low numbers of the 260/230 ratio does not impact downstream performance in the quantitative real-time PCR (qPCR) methods used.

To assess the quality of the extracted RNA the Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) was used. The Bioanalyzer estimates the integrity of the RNA by capillary electrophoresis, and gives a RNA integrity number (RIN) between 1 and 10, where 1 is the most degraded and 10 is the most intact profile. The algorithm used to calculate the RIN not only utilizes the ratio of 28S and 18S fragments, which has previously been used as a measurement of RNA degradation, but also takes into account several other factors that is indicative of RNA quality (Mueller *et al.* 2011), as shown in Figure 3.

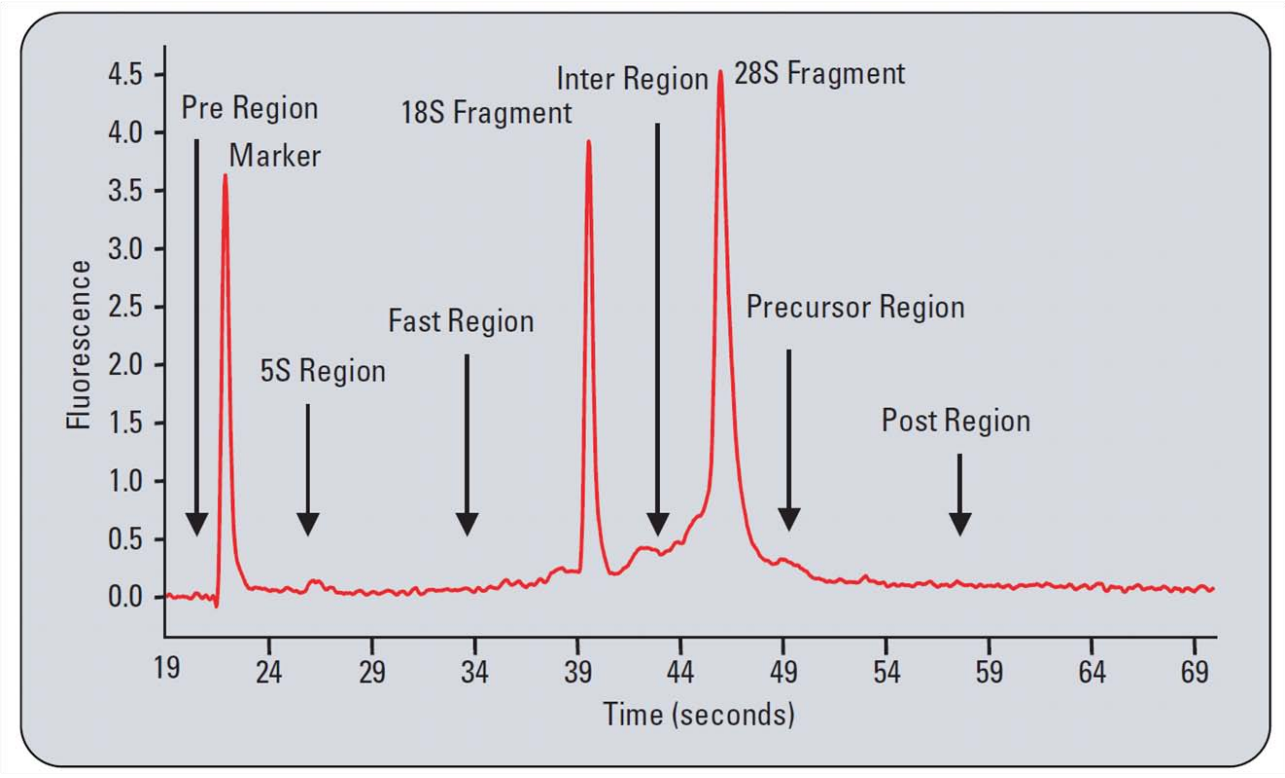

Figure 3: Factors indicative of RNA quality on the Agilent 2100 Bioanalyzer. Source: (Mueller *et al.* 2011).

The RIN vary greatly between tissues, which is possibly due to variations in connective tissues and lipid content. Samples with RINs above 5.0 are considered good quality RNA and RINs above 8 are perfect quality RNA (Fleige & Pfaffl 2006). However, it is generally agreed upon that if it is not possible to obtain high RINs, samples to be compared should at least exhibit uniform integrity values.

2.1.1.1 Purification of RNA from adipose tissue

In this thesis RNA was extracted from a variety of different pig tissues; liver, adipose tissues, small intestine, spleen, and mesenteric lymph nodes, using the phenol-chloroform method in the domestic pigs. RNA extraction from samples of all tissues, except for adipose tissues, were done with the column-based RNeasy Lipid Tissue Midi kit (Qiagen, Ballerup, Denmark) according to manufacturer's instructions (appendix 9.3), and extractions performed with this assay had high RINs and good NanoDrop results (see Table 4 and Table 5). However, when the same kit was applied to samples of abdominal SAT, the yields were very low. This could be due to the fact that the RNeasy Lipid Tissue Midi kit has an upper limit of 500 mg tissue, and the adipose tissue has a high lipid content and a low cell number. Therefore, the next step was to extract RNA from the abdominal SAT with the RNeasy Maxi kit (Qiagen) which has an upper limit of 1 g of tissue, according to manufacturer's instructions (appendix 9.4). This method proved successful for the samples of abdominal SAT in the pilot study for the pigs in group 1 (see Table 3, Table 5, and Paper I p. 27) with yields of 50-100 ng/µl and 260/230 and 260/280 ratios of about 2. However, when applied to the abdominal SAT samples of group 2 and 3 (Table 3), and to other adipose tissues, the method produced low yields of RNA. Therefore, variations of the method were tested, including higher concentrations of phenol/chloroform, changes in centrifugation (higher rpm and more time) and elution of RNA with more/less RNase-free water. However, none of this increased the RNA yields. Because of the low cell and high lipid content of adipose tissue, concerns were that most of the extracted RNA was retained in the membrane of the columns, so the next step was to circumvent the columns. Therefore it was necessary to find a way to precipitate the RNA without columns, and different proportions of ethanol and isopropanol was tried. This eventually gave good results with yields above 100 ng/µl, 260/230 ratios of around 2.3, 260/280 ratios of above 1.8 (see Table 5) and the method is described in detail in appendix 9.5, Paper I p. 27, and Paper II p. 39.

When applying this method to the adipose tissue samples from the Ossabaw and Göttingen minipigs, the method produced no yields of RNA. Another round of optimization was initiated; the amount of tissue was changed, new proportions of reagents were tested, whether ethanol was better than isopropanol to precipitate the RNA was tested, the temperature of the water used to elute was changed, and the centrifugation speed and time were changed. Although good yields were obtained during optimization on some samples, they had low 260nm/280nm ratios (about 1.6) and had poor performance when tested with qPCR. After good advice from Associate Professor Susanna Cirera (KU-SUND) another approach was tried as there was a possibility that the lipids interfered with the RNA extraction negatively; after the initial centrifugation of the homogenized sample with phenol in a cold centrifuge (4°C), the lipids in the sample had gathered in a layer on top. This lipid layer was discarded, and instead of air drying the RNA precipitate it was dried on a thermo block for 10 minutes at 55°C. This gave excellent results with yields of generally above 200 ng/µl and 260nm/280nm ratios of around 1.9 (Table 6 and Table 7). Therefore this method, as described in appendix p. 9.6, Paper III p. 70 and Paper IV p. 95, was used for extracting RNA from the adipose tissues of the minipigs. As the method was not tested on the domestic pigs it is not possible to account for its applicability in other species or tissues.

2.1.2 Real-time quantitative PCR (qPCR)

qPCR is a technique used to amplify and quantify a targeted DNA molecule. As qPCR is performed on double-stranded DNA molecules, it is necessary to convert mRNA into DNA before analysis can be performed. To do this the enzyme reverse transcriptase is used. Reverse transcriptase is a multifunctional enzyme with 3 distinct enzymatic activities: A RNA-dependent DNA polymerase that reverse transcribes complementary DNA (cDNA) from an RNA template, a hybrid-dependent exoribonuclease that degrades RNA hybridized to cDNA, and finally a DNA-dependent DNA polymerase that synthesizes the second cDNA strand. For all samples, the QuantiTect Reverse Transcription Kit (Qiagen) was used according to manufacturer's instructions. This kit includes a step that eliminates genomic DNA, which is important as this is one of the pitfalls of quantifying RNA by qPCR.

The next step is dependent on what PCR platform is to be used. For this thesis two platforms were used; the Rotor-Gene Q instrument (Qiagen) for analysis of a few genes or a few samples (method is described in appendix 9.8), and the high-throughput Bio-Mark (Fluidigm, CA, USA) for analysis of multiple genes (48/96) and samples (48/96) simultaneously (method is described in appendix 9.10). Before high-throughput qPCR, the samples were preamplified to stretch the samples (method is described in appendix 9.9).

Preamplification can be done by different methods; DNA-dependent DNA polymerase or DNA-dependent RNA polymerase, where antisense RNA is produced, which then is reverse transcribed into cDNA. In this thesis the first enzyme was used, via the TaqMan PreAmp Master Mix (Applied Biosystems, Nærum, Denmark) with 16 amplification cycles. The preamplification is done with the primers used for the high-throughput qPCR, to enrich the cDNA for gene specific targets.

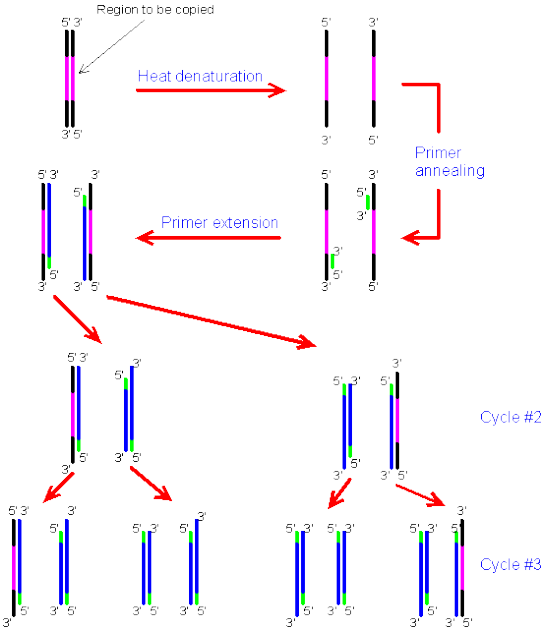

Figure 4: Schematic overview of the polymerase chain reaction. Primers are in green. Image obtained from: http://www.web-books.com/MoBio/Free/Ch9E.htm

PCR is a three-step process with denaturation, annealing and elongation steps, where the amount of cDNA is doubled by each reaction cycle (Freeman *et al.* 1999). The sequences of cDNA amplified are determined by the selection of primers. For detection and quantification of the amplified PCR product the dyes SYBR Green (SYBR green Jumpstart Taq Readymix, Sigma-Aldrich, MO, USA) and EvaGreen (EvaGreen 20X (Biotium, Inc., CA, USA) were used. These dyes bind to double-stranded DNA and produce a fluorescent signal. Furthermore, the Readymix and Master Mix (TaqMan Gene Expression Master mix, Applied Biosystems, CA, USA) contains an antibody that inactivates the DNA polymerase at room temperature. When the temperature is raised above 70°C in the first denaturation step the complex dissociates and the DNA polymerase becomes fully active.

The efficiency of the PCR is dependent on a variety of factors, including the annealing temperature and the length of the amplified product. The annealing temperature can vary greatly from primer to primer, which is not a problem in single-gene qPCR. However, when performing high-throughput qPCR, the annealing temperature will have to be the same for all primers, and this must be considered when designing primers.

Furthermore, the amplification of large products (>400 bp) is very dependent on good quality RNA, whereas short amplicons (70-250 bp) are less dependent of the quality of RNA (Fleige & Pfaffl 2006). In order to correct for sample to sample variations introduced in the up-stream procedures of qPCR, such as extraction yield, reverse-transcription yield and amplification efficiency, it is necessary to normalize the data to enable the comparison of mRNA concentrations across different samples. To do this, the expression of reference genes is measured. Reference genes are stable genes with abundance which show strong correlations with the amount of mRNA present in the sample. As the mRNA levels of reference genes can vary between tissues and individuals, it is important to validate each for the experimental setup, and to choose more than one reference gene (Bustin & Nolan 2004). GeNorm was used to determine the most stable reference genes. The program calculates a gene-stability measure (*M*) for all reference genes in a given set of samples, and thereby identifies the number and identity of the most stable reference genes in that set of samples. These reference genes are then used to calculate the geometric mean used to normalize the samples.

To accommodate the concerns raised as to what could negatively affect the reproducibility and biological relevance of the samples, several steps were taken. For the high-throughput qPCR, replicates were produced in the cDNA-synthesis, making it possible to check for introduced variation. For the single-gene qPCR, all samples were run in triplicates in the assay to check for pipetting variation. For both types of qPCR, no-template controls (NTCs) containing no sample and no reverse transcription (-RT) controls where the sample was not reverse transcribed were included in the assays to assess the degree of DNA contamination and specificity (e.g. primer dimers) of the results. Furthermore, 5 reference genes were included to be able to determine the optimal reference genes for normalization. To compensate for low quality RNA experienced in some samples, only primers with short amplicon targets (<250 bp) were used.

2.2 Protein expression in blood

To quantitatively determine the levels of specific proteins in blood serum, the method enzyme-linked immunosorbent assay (ELISA) was used. To be able to quantify the concentration of the protein, a standard curve based on known concentrations of antigen was prepared. ELISAs were performed to quantify the proteins ORM, haptoglobin, CRP, TNF-α, IL-6 and SAA. Two types of ELISAs were used in this work; the competitive ELISA (ORM) and the sandwich ELISA (the rest).

In the employed competitive ELISA format the plate is coated with antigen, and the sample and the competitive buffer with antibody is added. The more antibody that is present in the sample, the less antibody from the competitive buffer will be able to bind to the antigen-coated well. After addition of HRP-conjugated secondary antibody specific to the antibody from the competitive buffer, the amount of primary antibody bound to the well can be determined. The higher the concentration of antibody in the original sample, the lower the absorbance of the assay.

In the sandwich ELISA the plate is coated with antibody, and the sample is added. After addition of a HRP-conjugated antibody the amount of antigen bound can be measured. In the sandwich ELISA, the higher the concentration of antigen in the original sample, the higher the absorbance.

To accommodate introduced variation in the assays several steps were taken. In all the assays the standard curve was included, as well as wells without sample (blanks). The correlation of the standard curve was

estimated in each assay as a quality control, and the background contamination was calculated from the blanks. Furthermore, to accommodate variation introduced through pipetting, all samples were analyzed in duplicates.

3 Study I – Cloned/non-cloned lean/obese domestic pig

This study was done in collaboration with Aarhus University at Foulum, Tjele and was part of a large collaboration between different groups, universities and business funded by a FØSU-grant (Nutriomics – functional foods for cloned lean/obese pigs). All pigs were cloned and reared at Foulum. The object of the study was to generate a nutriomic pig model that would allow determination of the most important effects of food-derived interactions with pig gene expression, proteins and metabolites. This would make it possible to determine if cloned pigs were better nutritional models than non-cloned pigs (controls) in terms of less variation and equal responses as the controls, and to determine the effect of functional foods (pre- and probiotica, as well as hydrolyzed whey) on the response to obesity. So far, four papers have been published from this project (Rødgaard *et al.* 2012; Clausen *et al.* 2011; Christensen *et al.* 2012; Varga *et al.* 2010).

Unfortunately, several parts of the project did not go as according to plan. First, according to the project description it was estimated that the project would produce 120 clones of the domestic pig, as the lean phenotype (Figure 5A), and a further 120 clones of the obese phenotype pig. At time of completion for the cloning part of the project, 17 cloned domestic pigs and 10 cloned pigs of the obese phenotype were produced. Second, the supplier of the pre- and probiotics suddenly pulled out of the project, before their products had been investigated. Therefore, a new supplier was found, however, this time it was with hydrolyzed whey. Third, the choice of obese phenotype pig was problematic. At the start of the project, the Hungarian Mangalica (Mangalitsa) pig (see Figure 5C) was chosen as the obese phenotype pig because of availability. However, due to problems with cloning this breed it was abandoned for the Yucatan minipig

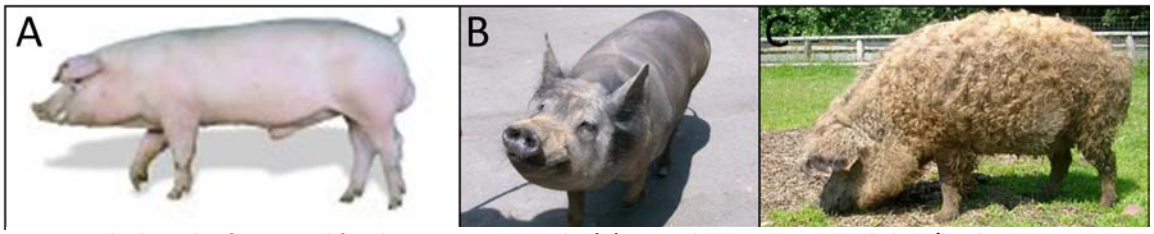

Figure 5: The breeds of pigs used for the Nutriomics study. (A) Lean phenotype pig: Yorkshire/landrace mix (domestic pig). (B) Obese phenotype pig: Yucatan minipig. (C) Obese phenotype pig: Hungarian Mangalica pig (not used).

(Figure 5B). The Hungarian Mangalica pig has not been investigated as an obese phenotype pig, and even though it has been used as a lard pig, and they are generally able to become very fat, there is no evidence that these pigs are negatively affected by their adiposity. Therefore, it is quite possible that this breed of pig, as other breeds of pigs, have developed ways to cope with the adiposity, making this pig not a very good model for obesity. In contrast, the Yucatan minipig has been used and investigated as a model for obesity and obesity-related diseases. However, most of these studies have been made in pigs bred selectively over several generations to have impaired glucose tolerance (discussed in (Bellinger *et al.* 2006)). In fact, when "normal" not-selectively bred Yucatan minipigs were investigated, they were found not to develop many of the comorbidities of obesity, including insulin resistance and atherosclerotic lesions (de Smet *et al.* 1998). The Yucatan minipigs bred for this study were not selectively bred. Because of this, the preliminary results that clones did not show less variation than controls, and the small groups of Yucatans produced (10 clones; 5 lean and 5 obese, and 7 controls; 4 lean and 3 obese), we chose not to use the Yucatan minipigs as an obese phenotype pig either in this project.

3.1 Background

The domestic pigs were bred over a 3 year period, starting January 2008 and ending January 2011. The 36 pigs used in this study were from three groups born almost a year apart. For specifications of the pigs, see Table 3, Paper I p. 27 and Paper II p. 39. For biochemical characteristics of the pigs, see appendix 9.12.

Group		Diets		Genetic composition
		Ad libitum	*Restricted*	
Group 1 (2008)	Clones	4		65% L 35% Y
	Controls	3		36% L 64% Y
		1		75% L 25% Y
Group 2 (2009)	Clones	5		65% L 35% Y
	Controls	6		75% L 25% Y
Group 3 (2010/2011)	Clones		8	65% L 35% Y
	Controls		9	75% L 25% Y
Total		**19**	**17**	

Table 3: Overview of the specifications of the domestic pigs used in Study I. The year in parenthesis indicates year of slaughter.

3.1.1 Cloning

Cloning has enormous potential applications; it can be used to generate multiple copies of transgenic animals for pharmaceutical production or xeno-transplantation, and to preserve endangered species. Furthermore, cloning is already an important tool for studying gene function, genomic re-programming, genetic diseases and gene therapy (discussed in (Tian *et al.* 2003)).

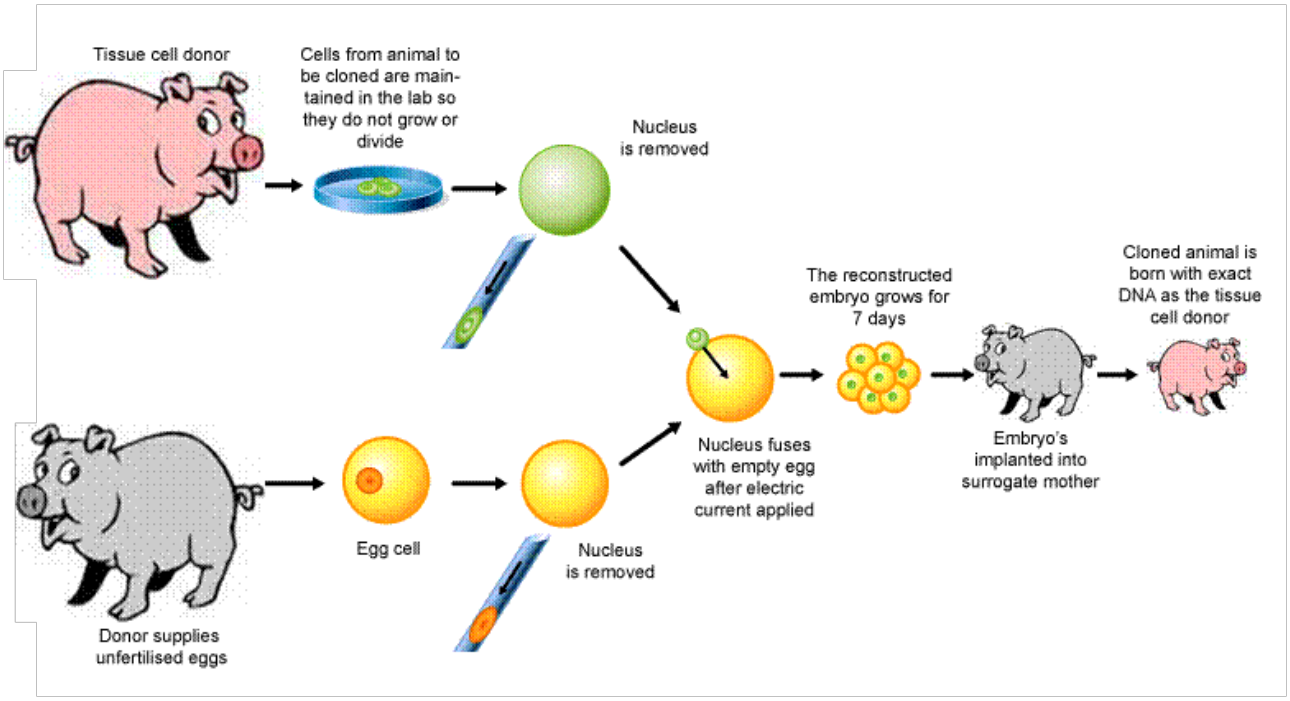

Figure 6: Cloning by somatic cell nuclear transfer (SCNT)

When cloning by somatic cell nuclear transfer (SCNT), the nucleus of a somatic cell is transferred into an enucleated oocyte from a donor (Figure 5). The genes inactivated through tissue differentiation are re-activated by the process of nuclear reprogramming, enabling the reversion of a differentiated nucleus into a totipotent cell. SCNT is widely used, and has been applied for this thesis.

Unfortunately, cloning by SCNT is a procedure very low in efficiency, with a success rate of only 0-4% (Wilmut *et al.* 2002), and a high percentage of embryo loss and perinatal mortality (Park *et al.* 2005; Whitworth *et al.* 2009). Even when born the cloned offspring has a higher mortality, and many die within the first 24 hours of birth (Wilmut *et al.* 2002). However, surviving cloned offspring generally have normal production and reproductive properties, even though cloned pigs have been showed to have deviant phenotypes. This is further described in Paper I p. 27 and Paper II p. 39.

One of the advantages of using cloned animals in biological investigation is to reduce the number of animals needed to obtain statistically sound data, as cloned animals are expected to show less inter-individual genetic and phenotypic variation due to identical genotypes. However, studies have repeatedly shown that cloned pigs do not have reduced variability, neither genetic nor phenotypic, when compared to normal outbred pigs. This is further described in Paper I p. 27 and Paper II p. 39.

3.1.2 Domestic pigs as model animals for nutritional studies

The domestic pig of Landrace/Yorkshire mix used for the Nutriomics study is a breed widely used as a production pig in Denmark. It has been bred selectively for leanness for more than 40 years. However, when fed a high cholesterol and high fat diet the domestic pigs develop humanoid atherosclerosis (Gerrity *et al.* 1979), and can with injections of streptozotocin, that induces DNA strands to break within β-cells, become hyperglycemic (Gerrity *et al.* 2001). Therefore, this breed of pig has been widely used as a model for diet-induced and diabetes-induced atherosclerosis (Gerrity *et al.* 1979; Gerrity *et al.* 2001; Askari *et al.* 2002; Natarajan *et al.* 2002). Furthermore, when fed a paleolithic diet, a diet consistent with the hunter-gatherer life-style of human ancestors, domestic pigs become leaner, have lower blood pressure and are more sensitive to insulin than pigs fed a cereal based diet, suggesting that pigs, as humans, are not adapted to a diet with large amounts of cereal (Jonsson *et al.* 2006).

3.2 Hypothesis for Study I

- Cloned animals show less inter-individual phenotypic variation, making it possible to reduce group numbers to retain statistical power in future studies
- Obesity alters the expression of innate immune response genes and the response of the innate immune genes is similar between cloned and control pigs, as pigs have similar genetic backgrounds
- The serum concentrations of innate immune response proteins are differentially expressed in obese pigs compared to lean and is similar between cloned and control pigs
- Adipose tissues from the abdomen; the abdominal SAT and VAT, have a higher number of affected innate immune response genes than the neck SAT

3.3 Results for Study I

3.3.1 Preliminary study of the expression of selected innate immune factors in 5 tissues

To help select tissues of interest, a small preliminary study of the pigs of Group 1 (4 clones and 4 controls, see Table 3) into the expression profiles of cytokines *IL1*, *IL6*, and *TNF* and APPs *HP*, *SAA* and *APOA1* was conducted with qPCR (Rotor-Gene) (described in appendix 9.8). The pre-PCR data is shown in Table 4.

Tissue	ng/µl	260/230	260/280	RIN
Liver	79-2419 (1257)	2.12-2.28 (2.22)	2.09-2.18 (2.14)	8.7-9.1 (8.9)
Abdominal SAT	74-191 (136)	1.93-2.19 (2.11)	2.08-2.17 (2.13)	7.1-8.7 (7.7)
Mesenteric lymphnodes	524-3152 (1680)	2.2-2.31 (2.26)	2.03-2.13 (2.08)	7.4-9.2 (8.7)
Spleen	1075-2054 (1370)	2.26-2.31 (2.29)	2.11-2.15 (2.12)	7.7-8.7 (8.3)
Small intestine	878-1805 (1361)	2.27-2.29 (2.29)	2.1-2.2 (2.15)	8.2-9.3 (8.9)

Table 4: Pre-qPCR data from the preliminary study. Yields, ratios and RINs of the pigs from Group 1 in the five measured tissues. The lowest and highest values are shown, with the mean in parenthesis. Small intestine n=7, the rest of the tissues n=8.

From a panel of five putative reference genes (beta-2-microglobulin (*B2M*), ribosomal protein L13A (*RPL13A*), *ACTB* (β-actin*)*, glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*) and hypozanthine phosphoribosyl transferase 1 (*HPRT1*)), the most stable (*RPL13A*, *GAPDH* and *HPRT1*) were selected using GeNorm. Figure 7 shows the melting curves, quantification curves and standard curve of a qPCR run with *RPL13A*, as a representative qPCR run on a Rotor-Gene Q instrument. The tissues investigated are possible immune regulatory sites, and were: liver (Figure 8A), spleen (Figure 8B), small intestine (Figure 8C), mesenteric lymph nodes (Figure 8D), and abdominal SAT (Figure 8E).

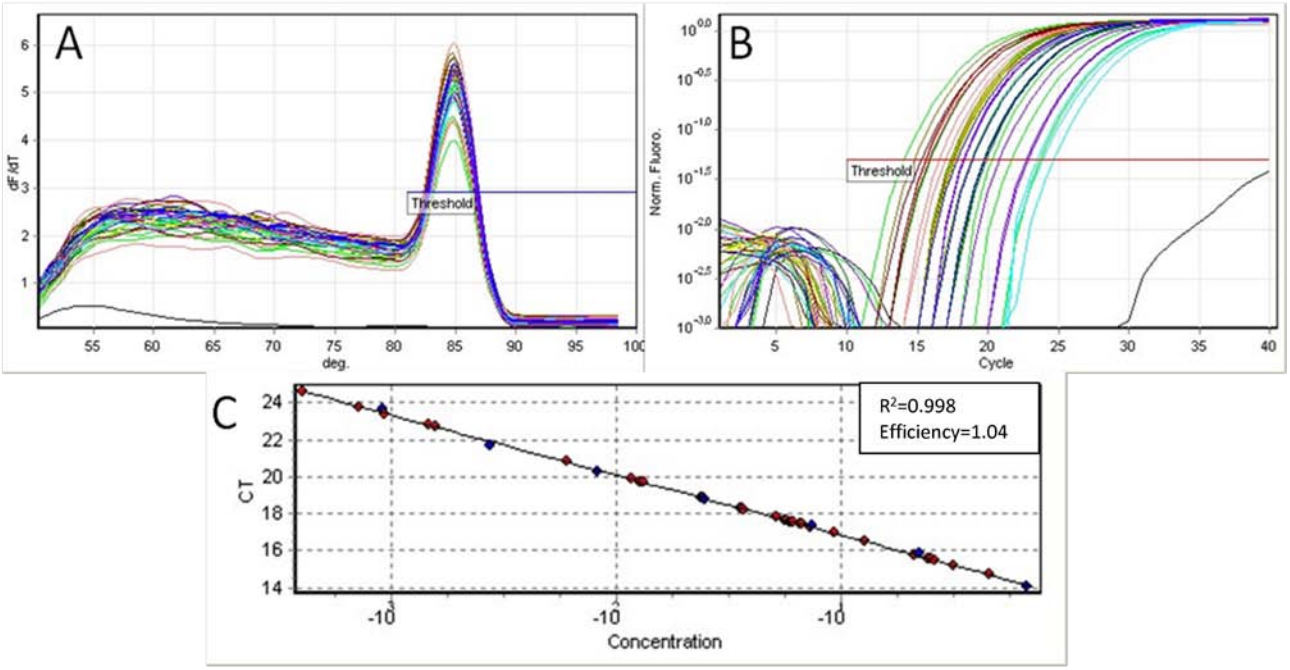

Figure 7: (A) Melting curves, (B) quantification curves and (C) standard curves from a representative run with *RPL13A*. Black line=NTC.

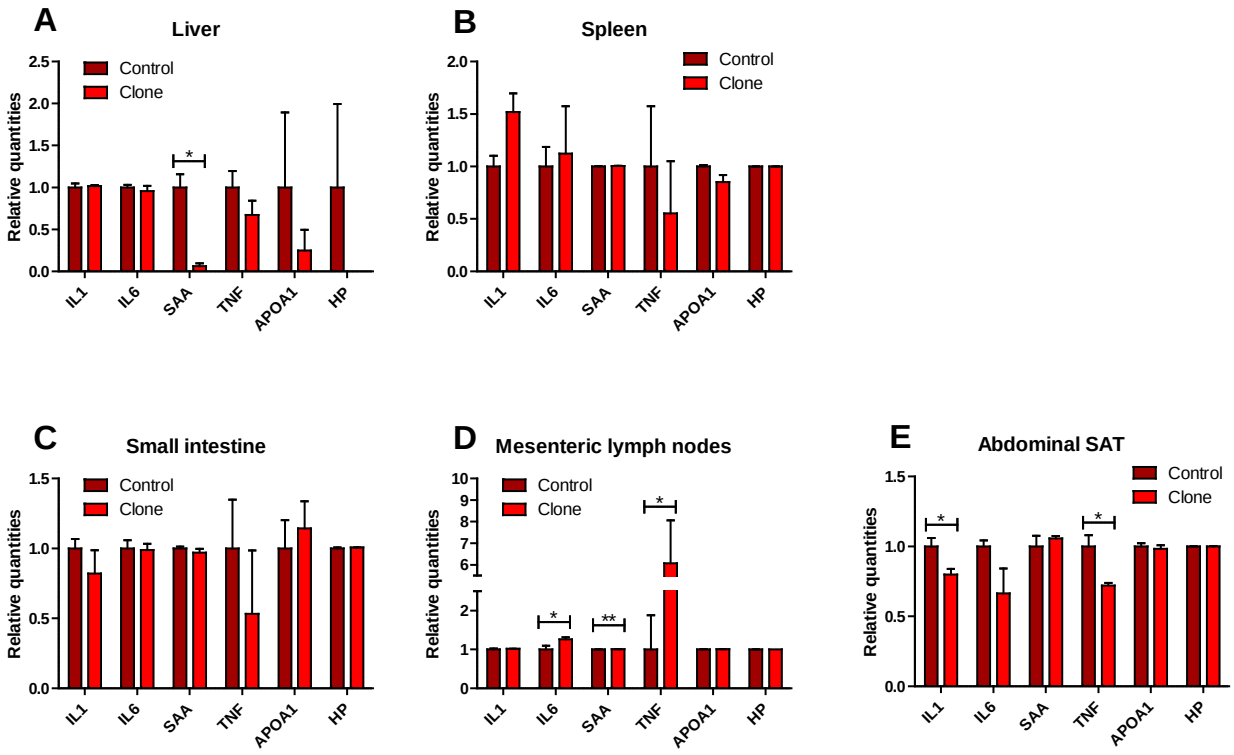

Figure 8: Expression of six innate immune regulatory genes in (A) liver, (B) spleen, (C) small intestine, (D) mesenteric lymph nodes and (E) abdominal SAT.

The geometric mean of the selected reference genes were used to normalize all samples. Relative expression for all samples were calculated relative to the least expressed sample for each primer assay, and data were log2 transformed to obtain a normal distribution prior to unpaired, two-tailed *t*-test. Data are expressed as the mean ± standard error of the mean (SEM).

Three of the tissues showed differential significant expression, namely the liver, mesenteric lymph nodes, and abdominal SAT (Figure 8). Therefore, the spleen and small intestine were not selected as tissue to investigate. Even though the liver only showed differential regulation of one of the 6 genes, this tissue was selected for further investigations as it is a way to gage the systemic reaction to obesity. Interestingly, the abdominal SAT showed differential significant regulation in two genes, and as the mesenteric lymph nodes, which drains the mesenteric VAT, among others, showed differential significant regulation in three genes, we decided to include the two adipose tissues (abdominal SAT and VAT) in the study. Furthermore, we decided to include the neck SAT as an adipose control tissue, as the expression is expected to be lower here than in the other two adipose tissues (as discussed previously).

This preliminary study was the first indication that the clones appeared to exhibit a different innate immune response pattern than the controls.

3.3.2 *Data from Study I not included in Paper I*

The yields and quality measurements of the 36 pigs and four tissues as measured with the NanoDrop and Bioanalyzer (discussed in section 2.1.1) are shown in Table 5. On average the yields, ratios and RINs of all the tissues were within accepted ranges.

Tissue	ng/µl	260/230	260/280	RIN
Liver	79-2419 (1078)	2.03-2.28 (2.22)	2.03-2.18 (2.12)	7.9-9.3 (8.8)
Abdominal SAT	60-734 (261)	1.92-2.55 (2.18)	1.69-2.17 (2.00)	6.8-9.3 (7.8)
VAT	42-600 (355)	1.66-2.62 (2.20)	1.58-2.19 (1.92)	5.1-9.4 (7.1)
Neck SAT	43-429 (109)	1.58-2.49 (2.22)	1.62-2.12 (1.85)	5.9-9.0 (7.4)

Table 5: Yields, ratios and RINs of the pigs from Study I in the four measured tissues. The lowest and highest values are shown, with the mean in parenthesis.

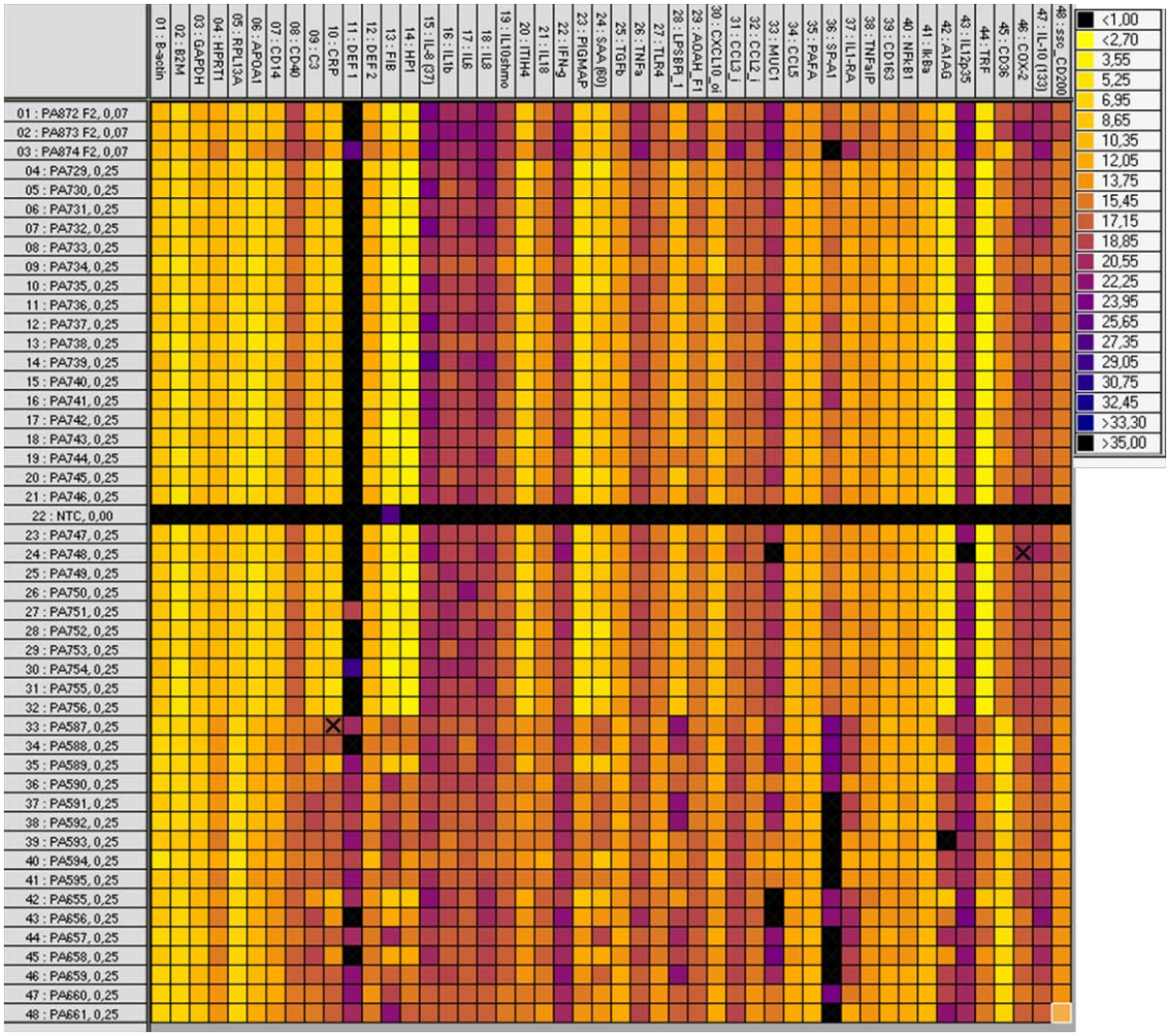

Figure 9: Heatmap from high-throughput qPCR (Fluidigm). On top the 48 primers are named and on the left side the 48 samples are shown. Samples from 01-03 is used to interrun calibrate the runs, samples from 04-32 (except 22, which is the NTC) is liver tissue samples, and samples from 33-48 is abdominal SAT samples.

The preliminary data of a qPCR run with a high-throughput Bio-Mark real-time PCR instrument (Fluidigm) depicted as a heatmap, is shown in Figure 9. The heatmap shown is from number four out of nine qPCR runs in total for Study I.

3.3.3 Paper I

Expression of innate immune response genes in liver and three types of adipose tissue in cloned pigs

Rødgaard T, Skovgaard K, Stagsted J, Heegaard PMH. Cell Reprogram. 2012, 14(5), Epub ahead of print

The gene expression of 40 innate immune response genes were investigated with high-throughput chip-based qPCR (FluiDigm) in liver, abdominal SAT, VAT and neck SAT, as well as the serum protein expression of six immune response factors with ELISA (haptoglobin, SAA, ORM1, CRP, IL-6 and TNF-α) in lean control and cloned domestic pigs. These pigs were not treated in any way, and the only difference between them was the cloning, making this a study into the effects of cloning. The results indicate subtle but distinct tissue specific changes in these four tissues upon cloning, with several genes being significantly differentially regulated in cloned domestic pigs. In the liver and the neck SAT (the two control tissues), the affected genes were generally upregulated in the clones, whereas, the affected genes were generally downregulated in the abdominal SAT and VAT. However, this did not translate into changed serum protein expression levels in the clones.

The variation between the clones and the controls were investigated as well, to see whether cloning results in less inter-individual variation. As the variance of the two groups was found to be equal, the variance was not reduced in the clones.

In conclusion, the fact that a notable number of genes of the innate immune system are affected by cloning could affect the usability of the cloned pigs as a model for nutritional studies negatively, and should be considered if choosing cloned pigs as a model for the study of the involvement in the innate immune system and inflammation in the development of diet-induced obesity-related diseases.

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Original Article

Expression of Innate Immune Response Genes in Liver and Three Types of Adipose Tissue in Cloned Pigs

Tina Rødgaard,[1] Kerstin Skovgaard,[1] Jan Stagsted,[2] and Peter M.H. Heegaard[1]

Abstract

The pig has been proposed as a relevant model for human obesity-induced inflammation, and cloning may improve the applicability of this model. We tested the assumptions that cloning would reduce interindividual variation in gene expression of innate immune factors and that their expression would remain unaffected by the cloning process. We investigated the expression of 40 innate immune factors by high-throughput quantitative real-time PCR in samples from liver, abdominal subcutaneous adipose tissue (SAT), visceral adipose tissue (VAT), and neck SAT in cloned pigs compared to normal outbred pigs.

The variation in gene expression was found to be similar for the two groups, and the expression of a small number of genes was significantly affected by cloning. In the VAT and abdominal SAT, six out of seven significantly differentially expressed genes were downregulated in the clones. In contrast, most differently expressed genes in both liver and neck SAT were upregulated (seven out of eight). Remarkably, acute phase proteins (APPs) dominated the upregulated genes in the liver, whereas APP expression was either unchanged or downregulated in abdominal SAT and VAT. The general conclusion from this work is that cloning leads to subtle changes in specific subsets of innate immune genes. Such changes, even if minor, may have phenotypic effects over time, *e.g.*, in models of long-term inflammation related to obesity.

Introduction

To elucidate the links between innate immune response gene expression and obesity, good animal models are needed. In general, pigs have a huge potential as biomedical models because of their human-like physiology and metabolic features. Specifically they should be uniquely useful as models for the obesity-induced inflammation-related host reactions thought to underlie development of disease related to obesity (the metabolic syndrome) (Litten-Brown et al., 2010). Although it is normally assumed that the use of cloned animals will exhibit less interindividual phenotypic variation and allow for a reduction of the number of animals needed to obtain statistically sound data, this assumption has been found not always to be warranted. For example, the variability in blood parameters and metabolites (Archer et al., 2003; Clausen et al., 2011) as well as the gene expression of fetal growth-related genes (Jiang et al., 2007) was found to be similar in cloned as in normal pigs. It has been proposed that some of this variation may stem from maternal mitochondrial DNA from the donor cell (St. John et al., 2005).

Previous work has shown that the production and use of cloned pigs is not straightforward because side effects inherent in currently employed cloning procedures may affect the phenotype of cloned pigs (Tian et al., 2008). Cloning by somatic cell nuclear transfer (SCNT) in pigs remains a low-efficiency process with a small proportion of live clones and increased mortality perinatally (Park et al., 2005; Whitworth et al., 2009). The increased mortality has been attributed to a range of causes, including metabolic and cardiopulmonary abnormalities (Hill et al., 1999; Wells et al., 1999), lymphoid hypoplasia (Renard et al., 1999), and neonatal respiratory distress (Hill et al., 1999). Interestingly, increased susceptibility of such cloned animals to bacterial infections has also been described repeatedly (Carter et al., 2002; Keefer et al., 2001; Peura et al., 2003). Surviving cloned pigs are generally found to be healthy and production and reproduction properties in adult cloned pigs are generally normal; however, there are several reports of cloned pigs having deviant phenotypes. These include pulmonary hypertension and other hemodynamic disorders, contracted foreleg tendons, and respiratory problems (Whyte et al., 2011), changes in

[1]Innate Immunology Group, National Veterinary Institute, Technical University of Denmark, 1870 Frederiksberg C, Denmark.
[2]Department of Food Science, Aarhus University, 8830 Tjele, Denmark.

metabolic phenotype (Christensen et al., 2012; Clausen et al., 2011), and a higher incidence of mild atelectasis (Park et al., 2011). Lower body weight is a common finding in cloned pigs, both at birth and later (Archer et al., 2003; Jiang et al., 2007; Park et al., 2011; Tian et al., 2008), although it is not always observed (Carter et al., 2002). Furthermore, subtle gene expression abnormalities may be accommodated by apparently normal phenotypes, as demonstrated in mice (Humpherys et al., 2001), with unpredictable consequences for more complex phenotypic traits. Park and co-workers (Park et al., 2011) analyzing gene expression in 1-month-old cloned piglets found most differently expressed genes showing decreased expression. We extend these studies here by performing a detailed study on the effect of cloning on the expression of innate immune response genes in liver and three types of adipose tissue in the pig.

Only a few studies have reported on the regulation of specific innate factors in cloned pigs. Thus, cloning was shown to downregulate the response to lipopolysaccharide (LPS) of proinflammatory cytokines [tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6)] in serum (Carroll et al., 2005), whereas another paper described the adaptive immune system of cloned pigs as being apparently not affected by the cloning process (Carter et al., 2002). Looking at baseline levels of as well as response to vaccination of several humoral and cellular adaptive immune parameters, Chavatte-Palmer and co-workers (2009) also found cloned heifers to be indistinguishable from control heifers.

There is increasing evidence that low-grade systemic inflammation is associated with obesity, linking obesity with the development of disease states related to inflammation, including atherosclerosis, type 2 diabetes, and cardiovascular disease (Bastard et al., 2006; Federico et al., 2010; Gil et al., 2007). Obesity leads to chronic activation of the innate immune system, resulting in a low-grade inflammation of the white adipose tissue. Important players of the innate immune system include cytokines, chemokines, and acute-phase proteins. Several of the proteins involved in innate and inflammatory responses have been found to be produced in adipose tissue, such as C-reactive protein (CRP), IL-6, and TNF-α (for review, see Fain, 2010; Fantuzzi, 2005), pointing to adipose tissue as being directly involved in maintaining or propagating low-grade inflammation as seen in obesity. Depot-specific differences in the gene expression profiles of adipose tissue [*e.g.*, visceral adipose tissue (VAT) as opposed to subcutaneous adipose tissue (SAT)] have been demonstrated in diabetic human subjects (Samaras et al., 2010) and in apparently healthy obese women (Alvehus et al., 2010). Furthermore, nonabdominal SAT has been found to be less metabolically active and to even offer protection against lipotoxicity and fat deposition (for review, see Wronska and Kmiec, 2012).

Therefore, we investigated the effect of cloning on expression of a range of key genes involved in innate immune reactions and/or inflammation in different types of relevant tissues in pigs cloned by the SCNT procedure focusing on the baseline expression at 9 months of age. To our knowledge, this is the first study on cloned pigs investigating innate immune response-related genes with relevance for obesity research in two types of adipose tissue from the abdominal region in addition to liver and neck SAT. In addition to demonstrating the feasibility of high-throughput quantitative PCR (qPCR) for analysis of expression of a large number of genes in different types of tissue, the results show that subtle changes occur in cloned animals as compared to noncloned animals in specific subsets of innate immune response genes in a tissue type–specific manner, serving as a reminder of some of the challenges inherent in using cloned model animals for biological and biomedical studies.

Materials and Methods

Animals, diets, and sampling

All experimental procedures involving animals were approved by the Danish Animal Experimental Committee. Cloning was performed at Aarhus University (Tjele, Denmark) using SCNT as described in Kragh et al. (2004). Donor cells were from cultured ear fibroblasts obtained from a Danish Landrace×Yorkshire (65%:35%) sow. The cloned embryos were surgically transferred to surrogate sows 5–6 days after cloning as described in Schmidt et al. (2010). Normal litters were used as controls (75% Danish Landrace:25% Yorkshire). They were obtained after standard artificial insemination. Cloned ($n=8$) and control ($n=9$) piglets were delivered normally. All pigs were reared in the same experimental stables of Aarhus University (Tjele, Denmark). All pigs were female.

Pigs were nursed by surrogate sows and weaned after 28 days. They were kept on a standard diet for an additional 2 months, after which they were individually housed and fed a high-energy diet (containing 10% sugar and 10% soy oil) restricted at 1.5 kg/day. At the time of slaughter, the average weight of the clones was 127.1 ± 5.9 kg and 119.1 ± 3.2 kg for the controls.

Pigs were killed at 9 months of age with a bolt pistol after overnight fasting. The animals were desangiunated, after which tissue samples from liver, abdominal fat (a combination of deep and superficial SAT), mesenteric fat surrounding the appendix (VAT), and subcutaneous fat from the neck (neck SAT) were obtained and snap frozen in liquid nitrogen. Blood was collected for serum preparation; it was allowed to clot at room temperature for 1 h, centrifuged at 3000 rpm for 10 min at 4°C, and then frozen. Tissue and serum samples were kept at −80°C until analysis.

Extraction of RNA

For RNA extracted from liver tissue the following method was used: Total RNA from ≈ 100 mg of liver tissue was isolated by a RNeasy Lipid tissue Midi kit (Qiagen, #75842) according to manufacturer's protocol. Samples were homogenized on a gentleMACS Dissociator (Miltenyi Biotec, Germany) in gentleMACS M tubes (Miltenyi Biotec, #130-093-458) and treated with on-column RNase-free DNase digestion (Qiagen, #79254).

A modified procedure was used for extraction of RNA from the VAT, abdominal SAT, and neck SAT. Total RNA from ≈ 1 g of fatty tissue was homogenized with QIAzol Lysis Reagent (1 mL of Lysis Reagent per 100 mg of tissue) in gentleMACS M tubes on the gentleMACS Dissociator. The tube with homogenate was left at room temperature for 5 min after which 2 mL of chloroform (Merck, #1.02445) was added. After shaking vigorously for 15 sec, the tube was left at room temperature for 2–3 min. The tube was then centrifuged at 4500 rpm for 20 min at 4°C. From the upper, aqueous phase

2.2 mL were carefully transferred to a new tube (Corning, NY, USA, #430828) and 1.4 mL of isopropanol (2-propanol, Merck, #1.09634) was added. The tube was placed at room temperature for 5 min and was then centrifuged at 4500 rpm for 10 min at 4°C. The supernatant was carefully aspirated and discarded. The pellet was washed 3 times in 10 mL of cold 75% ethanol (EtOH) and was centrifuged at 3300 rpm for 5 min at 4°C after each wash. The supernatant was removed completely and the pellet was left to air dry. Afterward, 50–100 μL of RNase-free water was added, and the tube was vortexed after 10 min at room temperature.

For both methods of RNA extraction, the RNA yield was measured on a NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies Inc., USA).

RNA integrity

For the assessment of RNA integrity, the Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) was used. The RNA integrity number (RIN) was determined using the Agilent RNA 6000 Nano Kit (Agilent Technologies, #5067-1511) according to the manufacturer's protocol. In the liver, all samples had a RIN of above 8, whereas all samples in the fatty tissues had a RIN of above 6, with the exception of VAT from one pig, which had a RIN of 5.1.

cDNA synthesis and preamplification

Two cDNA replicates were prepared from each sample of extracted RNA. Then 500 ng of total RNA was reverse transcribed using the QuantiTECT Reverse Transcription kit (Qiagen, #205311), containing a mix of random primers and oligo(dT), according to the manufacturer's instructions. cDNA was diluted 1:6 in low ethylenediaminetetraacetic acid (EDTA) Tris-EDTA (TE) buffer (VWR–Bie & Berntsen, Herlev) prior to preamplification. Preamplification was performed using TaqMan PreAmp Master Mix (Applied Biosystems, #PN 4391128). Stocks of 200 nM primer mix were prepared combining equal concentration of all primers used in the present study (see Table 1). TaqMan PreAmp Master Mix (5 μL) was mixed with 2.5 μL of 200 nM stock primer mix and 2.5 μL of diluted cDNA, and incubated at 95°C for 10 min followed by 16 cycles of 95°C for 15 sec and 60°C for 4 min. Preamplified cDNA was diluted at least 1:4 in low EDTA TE buffer before qPCR.

Primer design and validation

Primers were designed using Primer3 (http://frodo.wi.mit.edu/) as described previously (Skovgaard et al., 2009) and synthesized at TAG Copenhagen (Copenhagen, Denmark). Primer sequences and amplicon lengths are shown in Table 1. Primer amplification efficiencies and dynamic range were acquired from three individual standard curves constructed from dilution series of highly responding samples. To ensure primer specificity, melting curves were inspected for all primer assays, and agarose gel electrophoresis and sequencing were performed for selected primer assays.

Quantitative real-time PCR

qPCR was performed in 48.48 Dynamic Array Integrated Fluidic Circuits (Fluidigm, CA, USA), combining 48 samples with 48 primer sets for 2304 simultaneous qPCR reactions. The reaction mix was prepared using the following components for each of the 48 sample reactions: 3 μL of ABI TaqMan Gene Expression Master Mix (Applied Biosystems), 0.3 μL of 20×DNA Binding Dye Sample Loading Reagent (Fluidigm), 0.3 μL of 20×EvaGreen (Biotium, VWR–Bie & Berntsen), and 0.9 μL of low EDTA TE buffer. Reaction mix (4.5 μL) was mixed with 1.5 μL of preamplified cDNA, diluted at least 1:4 in low EDTA TE buffer. Specific primer mixes for each of the 48 primer sets were then prepared using 2.3 μL of 20 μM primer (forward and reverse), 2.5 μL of 2×Assay Loading Reagent (Fluidigm), and 0.2 μL of low EDTA TE buffer. Reaction mix, including cDNA (5 μL) and primer mix (5 μL), was dispensed into appropriate inlets and loaded into the integrated fluidic circuit of the Dynamic Array in the IFC Controller (Fluidigm). After loading and mixing of each sample–primer reaction in individual chambers, the Dynamic Array was placed in the BioMark real-time PCR instrument (Fluidigm) and the following cycle parameters were used: 2 min at 50°C, 10 min at 95°C, followed by 35 cycles with denaturing for 15 sec at 95°C and annealing/elongation for 1 min at 60°C. Melting curves were generated after each run to confirm a single PCR product (from 60°C to 95°C, increasing 1°C/3 sec). Nontemplate controls (NTC) were included to indicate potential problems with nonspecific amplification or sample contaminations. Expression data were acquired using the Fluidigm Real-Time PCR Analysis software 3.0.2 (Fluidigm) and exported to GenEx5 (MultiD, Göteborg, Sweden).

Data analysis and statistics

Data preprocessing, normalization, relative quantification, and statistics were performed using GenEx5. Data were corrected for PCR efficiency for each primer assay individually. The most stably expressed reference genes [for liver, hypoxanthine phosphoribosyl transferase 1 (*HPRT1*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and for adipose tissues, *HPRT1* and β-2-microglobulin (*B2M*)] were identified from a panel of five putative reference genes using GeNorm (Vandesompele et al., 2002), and the geometric means of the most stably expressed genes were used to normalize all samples in GenEx5. Most stably expressed reference genes were determined for each tissue type individually as well as for the combined dataset. To visualize differential gene expression, relative expression for all samples was calculated relative to the least expressed samples for each primer assay. Data were $\log_2$ transformed to attain a normal distribution prior to *t*-test, F-test, and analysis of variance (ANOVA). Gene expression changes were considered to be significant if the *p* value was equal to or less than 0.05 and a fold change of ±1.5 was arbitrarily defined as the cutoff for biologically significant changes. Data are expressed as the mean ± standard error of the mean (SEM).

Quantitative serum enzyme-linked immunosorbent assay

Serum concentration of CRP was analyzed by a sandwich-type enzyme-linked immunosorbent assay (ELISA) using dendrimer-coupled cytidine diphosphocholine (a CRP-binding ligand) in the coating layer, as described in Heegaard et al. (2009), employing polyclonal rabbit anti-human antibodies with cross-reactivity toward porcine CRP followed by

TABLE 1. GENES, PRIMER SEQUENCES, AND AMPLICON LENGTHS USED FOR HIGH-THROUGHPUT qPCR

Gene symbol	*Gene name*	*Sequence*	*Amplicon length*
AOAH	Acyloxyacyl hydrolase	F: GTAATGGCATTTGGGGTGTC R: TCTCCCAGCAAAATGATTCC	97
ORM1	Orosomucoid 1	F: AGTCCTGAGCCTCCTTCCTC R: GCCGAGCCGATATAATACCA	123
APOA1	Apolipoprotein A-1	F: GTTCTGGGACAACCTGGAAA R: GCTGCACCTTCTTCTTCACC	86
B2M	β-2-microglobulin	F: TGAAGCACGTGACTCTCGAT R: CTCTGTGATGCCGGTTAGTG	70
ACTB	β-actin	F: CTACGTCGCCCTGGACTTC R: GCAGCTCGTAGCTCTTCTCC	76
CRP	C-reactive protein	F: GGTGGGAGACATTGGAGATG R: GAAGGTCCCACCAGCATAGA	85
CD14	CD14	F: GGGTTCCTGCTCAGATTCTG R: CCCACGACACATTACGGAGT	164
CD36	CD36	F: GCCTATCCTCTGGCTTAATGAG R: AACATCCCCACACCAACACT	135
CD40	CD40	F: TGAGAGCCCTGGTGGTTATC R: GCTCCTTGGTCACCTTTCTG	90
CD163	CD163	F: CACATGTGCCAACAAAATAAGAC R: CACCACCTGAGCATCTTCAA	130
CD200	CD200	F: TCCCCAGGAAGTTTTGATTG R: CCATGGTTCTTGCTGAAGGT	84
CCL2	Chemokine (C-C motif) ligand 2	F: GCAAGTGTCCTAAAGAAGCAGTG R: TCCAGGTGGCTTATGGAGTC	103
CCL3L1	Chemokine (C-C motif) ligand 3-like 1	F: CCAGGTCTTCTCTGCACCAC R: GCTACGAATTTGCGAGGAAG	90
CCL5	Chemokine (C-C motif) ligand 5	F: CTCCATGGCAGCAGTCGT R: AAGGCTTCCTCCATCCTAGC	121
CXCL10	Chemokine C-X-C motif ligand 10	F: CCCACATGTTGAGATCATTGC R: GCTTCTCTCTGTGTTCGAGGA	141
C3	Complement component 3	F: ATCAAATCAGGCTCCGATGA R: GGGCTTCTCTGCATTTGATG	76
DEFB1	Defensin, β 1	F: ACCTGTGCCAGGTCTACTAAAAA R: GGTGCCGATCTGTTTCATCT	109
PBD2	Defensin, β 2	F: CAGGATTGAAGGGACCTGTT R: CTTCACTTGGCCTGTGTGTC	99
FIB	Fibrinogen	F: GAATTTTGGCTGGGAAATGA R: CAGTCCTCCAGCTGCACTCT	86
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F: ACCCAGAAGACTGTGGATGG R: AAGCAGGGATGATGTTCTGG	79
HP	Haptoglobin	F: ACAGATGCCACAGATGACAGC R: CGTGCGCAGTTTGTAGTAGG	105
HPRT1	Hypoxanthine phosphoribosyl transferase 1	F: ACACTGGCAAAACAATGCAA R: TGCAACCTTGACCATCTTTG	71
IL1B	Interleukin-1β	F: CCAAAGAGGGACATGGAGAA R: GGGCTTTTGTTCTGCTTGAG	123
IL6	Interleukin-6	F: TGGGTTCAATCAGGAGACCT R: CAGCCTCGACATTTCCCTTA	116
IL8(a)	Interleukin-8	F: GAAGAGAACTGAGAAGCAACAACA R: TTGTGTTGGCATCTTTACTGAGA	99
IL8(b)	Interleukin-8	F: TTGCCAGAGAAATCACAGGA R: TGCATGGGACACTGGAAATA	78
IL10(a)	Interleukin-10	F: CTGCCTCCCACTTTCTCTTG R: TCAAAGGGGCTCCCTAGTTT	95
IL10(b)	Interleukin-10	F: TACAACAGGGGCTTGCTCTT R: GCCAGGAAGATCAGGCAATA	110
IL12A	Interleukin-12A	F: CCACCTGGACCATCTCAGTT R: CAGCAGATTTTGGGAGTGGT	94
IL18	Interleukin-18	F: CTGCTGAACCGGAAGACAAT R: TCCGATTCCAGGTCTTCATC	100
IL1RN	Interleukin-1 receptor antagonist	F: TGCCTGTCCTGTGTCAAGTC R: GTCCTGCTCGCTGTTCTTTC	90

(*continued*)

TABLE 1. (CONTINUED)

Gene symbol	*Gene name*	*Sequence*	*Amplicon length*
ITIH4(b)	Inter-α-trypsin inhibitor heavy- chain family, member 4	F: AGGCCCTCACCATATCACAG R: GTTGCCATCCAGGACTGTTT	110
ITIH4(a)	Inter-α-trypsin inhibitor heavy- chain family, member 4	F: ATGACAGCAAGCGAACAGTG R: GGGGATCCCTCTTGGTAATC	85
IFNG	Interferon-γ	F: CCATTCAAAGGAGCATGGAT R: TTCAGTTTCCCAGAGCTACCA	76
LBP	Lipopolysaccharide binding protein	F: CCCAAGGTCAATGATAAGTTGG R: ATCTGGAGAACAGGGTCGTG	83
MUC1	Mucin	F: GGATTTCTGAATTGTTTTTGCAG R: ACTGTCTTGGAAGGCCAGAA	116
NFKB1	Nuclear factor of kappa light polypeptide enhancer in B-cells 1	F: CTCGCACAAGGAGACATGAA R: GGGTAGCCCAGTTTTTGTCA	97
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	F: GAGGATGAGCTGCCCTATGAC R: CCATGGTCTTTTAGACACTTTCC	85
PAFAH1B1	Platelet-activating factor acetylhydrolase 1b	F: GCAAACTGGCTACTGTGTGAAG R: GCACAGTCTGGTCATTGGAA	113
COX2	Prostaglandin-endoperoxide synthase 2	F: AGGCTGATACTGATAGGAGAAACG R: GCAGCTCTGGGTCAAACTTC	100
RPL13A	Ribosomal protein L13a	F: ATTGTGGCCAAGCAGGTACT R: AATTGCCAGAAATGTTGATGC	76
SAA	Serum amyloid A	F: TGGAGAGCCTACTCGGACAT R: CCTTTGGGCAGCATCATAGT	90
SFTPA1	Surfactant protein A1	F: CATGGGTGTCCTCAGTTTCC R: CATCAAAAGCGACTGACTGC	86
TLR4	Toll-like receptor 4	F: TTTCCACAAAAGTCGGAAGG R: CAACTTCTGCAGGACGATGA	145
TF	Transferrin	F: CTCAACCTCAAAACTCCTGGAA R: CCGTCTCCATCAGGTGGTA	82
TGFB1	Transforming growth factor beta 1	F: GCAAGGTCCTGGCTCTGTA R: TAGTACACGATGGGCAGTGG	97
TNF	Tumor necrosis factor	F: CCCCCAGAAGGAAGAGTTTC R: CGGGCTTATCTGAGGTTTGA	92
TNFAIP3	Tumor necrosis factor-α-induced protein 3	F: CCCAGCTTTCTCTCATGGAC R: TTGGTTCTTCTGCCGTCTCT	113

F, forward; R, reverse.

peroxidase-conjugated goat anti-rabbit antibody for detection (both antibodies from DAKO, Glostrup, Denmark). The cross-reactivity of the anti-human CRP antibody with pig CRP was demonstrated previously (Heegaard et al., 1998), and, in combination with catching of CRP by diphosphocholine, specificity is ensured for CRP. Pooled pig serum calibrated against a human CRP calibrator (DAKO A0073) was used as standard. The detection limit was 0.067 μg/mL (human equivalents).

Serum concentration of haptoglobin (HP) was determined by a sandwich ELISA using an in-house mouse anti-porcine HP monoclonal antibody in the coating layer and biotinylated commercial rabbit anti-human HP (DAKO P397) as the detection antibody, as described previously (Sorensen et al., 2006), with a lower limit of quantification of 1.4 μg/mL.

Orosomucoid (ORM) was analyzed by a competitive catching ELISA in which a mouse monoclonal antibody specific for ORM [1.62, prepared in-house (Heegaard et al., in preparation)], was used as the catching antibody in the coating layer. This was followed by simultaneous incubation with sample and biotinylated ORM (50 μL of each in the same well). Pooled pig serum calibrated against an ORM calibrator (Saikin Kagaku Institute Co. Ltd., Japan) was used as a standard. The detection limit of the assay was 50 μg/mL).

Commercially available sandwich ELISAs were used to determine serum IL-6 (Porcine IL-6 Duoset kit, R&D Systems, inc. #DY686), serum TNF-α (Swine TNF-α CytoSet™, Invitrogen, CA, USA, #CSC1753 with Antibody Pair Buffer Kit, Invitrogen, CA, USA, #CNB0011), and serum amyloid A (SAA; Phase SAA assay, Tridelta Development Ltd., Kildare, Ireland, #TP 802). Samples were tested according to manufacturer's instructions. The lower limits of quantification were defined as 62 pg/mL for the IL-6 assay, 126 pg/mL for the TNF-α assay, and 6.25 μg/mL for the SAA assay.

The plates were developed using an orthophenylenediamine peroxide procedure (TMB-PLUS, Kem-En-Tec, #4390A) according to the manufacturer's instructions. The optical densities of wells were read at 450 nm, and unspecific coloration was subtracted at 650 nm (550 nm for IL-6 ELISA assay) using an automatic plate reader (Thermo Multiskan

Ex spectrophotometer, Thermo Scientific, Waltham, MA, USA). All samples including standard were analyzed in duplicates. For all assays, an intraassay coefficient of variation of <15% was accepted. Sample values were calculated from the curve fitted to the readings of the standard (using Ascent software v. 2.6, Thermo Scientific). Significance was tested using a two-tailed unpaired *t*-test, and variance was tested with an F-test. Changes in concentrations were considered to be significant if the p value was equal to or less than 0.05. Data from HP, CRP, and ORM ELISAs were evaluated with the Grubbs outlier test (Grubbs, 1969), and outliers were removed from dataset. Data are expressed as the mean ± SEM.

Results

Pigs

There was no significant difference between the body weights of cloned and control pigs ($p = 0.24$). Additional metabolic data for these pigs is published elsewhere (Christensen et al., 2012).

Gene expression

The expression of 43 genes related to innate immunity, as well as five reference genes, was investigated. The following genes were removed due to low efficiencies; mucin (*MUC1*), defensin beta 1 (*DEFB1*), *IL12A* (all tissues), and surfactant protein A1 (*SFTPA1*) (all adipose tissues). Additional genes were removed in the quality validation of the technical replicates (cDNA), if the standard deviation was above 15%; in the liver, *IL8(a)* and *IL6* were removed and in the adipose tissues, fibrinogen (*FIB*), interferon gamma (*IFNG*), LPS binding protein (*LBP*), IL-1 receptor antagonist (*IL1RN*), *ORM1*, and *IL10(b)* were removed. *FIB* and *ORM1* were measurable in the liver but was over detection limit in the adipose tissues.

Out of the remaining 40 genes, the ones showing statistically significant differences and a fold change of at least ±1.5

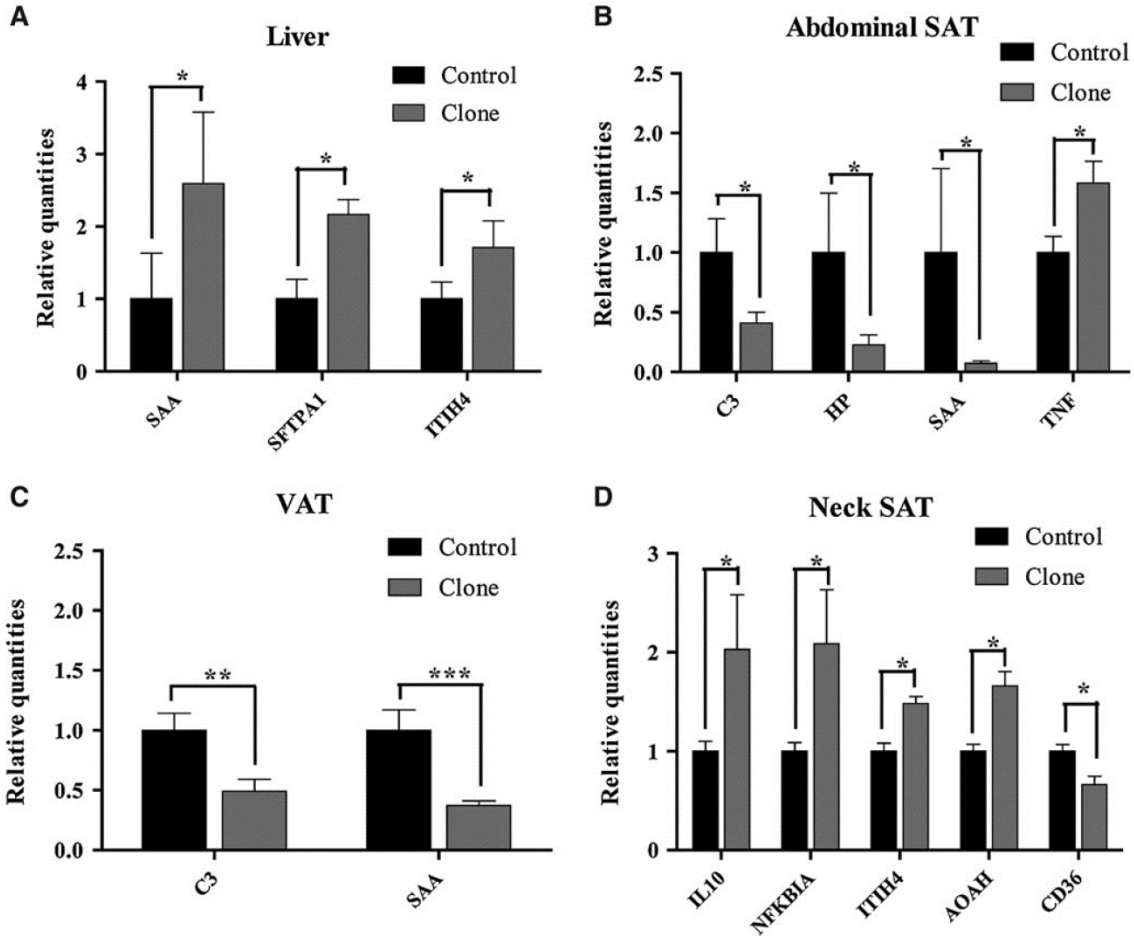

FIG 1. Significantly differently expressed genes in cloned as compared to control pigs (=1) with a fold change of ±1.5 in liver (**A**), abdominal SAT (**B**), VAT (**C**), and neck SAT (**D**) as measured with qPCR. For all tissues, controls $n = 9$ and clones $n = 8$ ($n = 7$ in neck SAT). Error bars depict SEM. (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$.

in the gene expression are shown in Figure 1. Expression levels for genes were normalized relative to control pigs (set to 1). Table 2 lists a complete overview of the relative gene expression in cloned compared to control pigs.

Gene expression patterns compared between control and cloned pig groups revealed minor changes in all four tissues in the expression of a few of the genes in the cloned pigs (Fig. 1).

In both of the two adipose tissues from the abdominal region [abdominal SAT (Fig. 1B) and VAT (Fig. 1C)], a general tendency for downregulation of genes in the cloned pigs was observed (Table 2). In the abdominal SAT, cloned pigs showed downregulated expression of the acute-phase proteins (APPs) *HP* and *SAA* as well as complement component 3 (*C3*). In contrast, the general trend for the differently expressed genes in cloned pigs both in the liver (Fig. 1A) and nonabdominal adipose tissue (neck SAT) (Fig. 1D) was to be upregulated; seven out of eight significantly differently expressed genes being upregulated in these two tissues.

In the liver (Fig. 1A), three out of the 40 genes investigated were upregulated significantly in the cloned pigs, but no genes were significantly downregulated in the cloned pigs compared to controls. Thus, the liver, representing a non-adipose, systemic organ, shows a quite stable phenotype, the only exception being upregulated expression of some APPs and the surfactant protein *SFTPA1*, a collectin involved in defense against bacteria, in the clones. Interestingly, this was not accompanied by increases in the expression of the proinflammatory cytokines included in the analysis; IL-1β

Table 2. Relative gene Expression (mRNA levels) in Cloned and Control Pigs

	Liver		*Abdominal SAT*		*VAT*		*Neck SAT*	
	Clone	*Control*	*Clone*	*Control*	*Clone*	*Control*	*Clone*	*Control*
APOA1	1.05±0.05	1.00±0.10	1.23±0.18	1.00±0.11	1.38±0.13	1.00±0.19	1.35±0.19	1.00±0.05
CD14	0.96±0.04	1.00±0.08	0.76±0.09	1.00±0.11	0.91±0.11	1.00±0.14	0.80±0.08	1.00±0.06
CD40	1.04±0.15	1.00±0.09	0.76±0.08	1.00±0.12	0.62±0.04	1.00±0.33	1.26±0.11	1.00±0.10
C3	1.02±0.08	1.00±0.11	0.41±0.09	1.00±0.27	0.49±0.11	1.00±0.13	0.95±0.13	1.00±0.29
CRP	1.67±0.76	1.00±0.36	1.06±0.10	1.00±0.11	0.59±0.05	1.00±0.44	0.73±0.11	1.00±0.27
PDB2	0.92±0.28	1.00±0.39	0.64±0.10	1.00±0.21	0.91±0.26	1.00±0.24	2.62±1.26	1.00±0.13
HP	1.28±0.11	1.00±0.12	0.23±0.09	1.00±0.47	0.20±0.03	1.00±0.61	0.92±0.63	1.00±0.45
IL1B	0.89±0.18	1.00±0.14	1.65±0.48	1.00±0.27	0.80±0.30	1.00±0.38	2.07±0.79	1.00±0.20
IL18	0.90±0.08	1.00±0.08	0.67±0.13	1.00±0.20	0.69±0.18	1.00±0.35	2.13±1.02	1.00±0.10
COX2	1.63±0.37	1.00±0.15	1.63±0.40	1.00±0.19	1.33±0.32	1.00±0.18	2.66±1.72	1.00±0.19
SAA	2.59±0.98	1.00±0.63	0.07±0.02	1.00±0.66	0.37±0.04	1.00±0.15	1.27±0.19	1.00±0.17
TGFB1	0.88±0.08	1.00±0.10	0.82±0.05	1.00±0.06	0.75±0.07	1.00±0.12	1.03±0.09	1.00±0.05
TNF	1.25±0.29	1.00±0.20	1.58±0.20	1.00±0.13	1.02±0.17	1.00±0.24	1.61±0.36	1.00±0.14
FIB	1.18±0.05	1.00±0.12	ODL	ODL	ODL	ODL	ODL	ODL
ORM1	1.10±0.06	1.00±0.08	ODL	ODL	ODL	ODL	ODL	ODL
CD36	0.99±0.13	1.00±0.04	1.24±0.20	1.00±0.21	1.26±0.07	1.00±0.15	0.66±0.10	1.00±0.06
TLR4	0.87±0.11	1.00±0.12	1.15±0.17	1.00±0.16	1.17±0.18	1.00±0.10	1.05±0.12	1.00±0.03
AOAH	1.17±0.17	1.00±0.09	1.29±0.13	1.00±0.11	1.35±0.13	1.00±0.17	1.66±0.17	1.00±0.06
CXCL10	2.72±1.77	1.00±0.10	1.99±1.19	1.00±0.27	3.04±2.37	1.00±0.21	5.88±5.18	1.00±0.14
CCL3L1	1.11±0.23	1.00±0.12	1.22±0.12	1.00±0.16	0.76±0.14	1.00±0.40	0.96±0.16	1.00±0.20
CCL2	1.06±0.26	1.00±0.26	0.84±0.08	1.00±0.13	0.60±0.14	1.00±0.20	1.10±0.34	1.00±0.14
CCL5	0.99±0.06	1.00±0.08	1.01±0.06	1.00±0.21	1.50±0.26	1.00±0.11	1.40±0.15	1.00±0.12
PAFAH1B1	0.91±0.04	1.00±0.08	0.87±0.05	1.00±0.03	0.96±0.07	1.00±0.07	0.76±0.03	1.00±0.02
TNFAIP3	1.02±0.10	1.00±0.11	1.21±0.14	1.00±0.09	1.14±0.21	1.00±0.16	1.14±0.16	1.00±0.06
CD163	0.94±0.19	1.00±0.19	1.22±0.16	1.00±0.08	1.16±0.14	1.00±0.12	1.53±0.20	1.00±0.12
SFTPA1	2.16±0.21	1.00±0.27	NQ	NQ	NQ	NQ	NQ	NQ
IL1RN	0.99±0.08	1.00±0.09	NQ	NQ	NQ	NQ	NQ	NQ
CD200	0.93±0.08	1.00±0.08	0.72±0.11	1.00±0.14	0.97±0.08	1.00±0.16	0.85±0.17	1.00±0.13
IFNG	0.88±0.14	1.00±0.07	NQ	NQ	NQ	NQ	NQ	NQ
TF	1.11±0.03	1.00±0.09	0.49±0.25	1.00±0.67	0.11±0.03	1.00±0.79	1.54±1.06	1.00±0.34
NFKBIA	1.18±0.06	1.00±0.07	1.28±0.17	1.00±0.11	0.97±0.11	1.00±0.15	2.09±0.62	1.00±0.08
LBP	1.46±0.30	1.00±0.20	NQ	NQ	NQ	NQ	NQ	NQ
IL10(a)	1.36±0.34	1.00±0.13	1.13±0.15	1.00±0.11	1.17±0.19	1.00±0.11	2.03±0.63	1.00±0.09
IL10(b)	1.71±0.55	1.00±0.14	NQ	NQ	NQ	NQ	NQ	NQ
NFKB1	0.93±0.05	1.00±0.05	0.83±0.07	1.00±0.07	0.84±0.05	1.00±0.06	0.95±0.13	1.00±0.02
IL6	NQ	NQ	2.04±0.74	1.00±0.36	0.73±0.21	1.00±0.26	1.19±0.67	1.00±0.20
ITIH4(b)	1.61±0.34	1.00±0.22	1.46±0.34	1.00±0.09	1.21±0.16	1.00±0.20	1.25±0.17	1.00±0.08
ITIH4 (a)	1.71±0.37	1.00±0.23	1.44±0.27	1.00±0.10	0.87±0.16	1.00±0.24	1.48±0.08	1.00±0.07
IL8(a)	NQ	NQ	0.58±0.13	1.00±0.38	0.44±0.10	1.00±0.50	3.98±3.28	1.00±0.28
IL8(b)	0.92±0.23	1.00±0.15	0.57±0.13	0.49±0.13	3.11±2.12	1.00±0.40	1.00±0.45	1.00±0.30

Relative innate immune gene expression in liver, abdominal SAT, VAT, and neck SAT in cloned pigs relative to control pigs (=1) as analyzed by qPCR±SEM.

SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue; ODL, over detection limit; NQ, not quantifiable; qPCR, quantitative PCR; SEM, standard error of the mean.

(*IL1B*), TNF-α (*TNF*), *IL8*, and *IL6* (not quantifiable in the liver); however, in the abdominal SAT (Fig. 1B) of clones *TNF* was indeed significantly upregulated, and *IL1B* and *IL6* were borderline significantly upregulated, with more than a 1.5-fold increase compared to the level of control pigs (Table 2).

Cloning appeared to affect gene expression in VAT (Fig. 1C) in much the same way as in abdominal SAT, with *SAA* and *C3* being significantly downregulated and *HP* being borderline significantly downregulated with a five-fold decrease compared to the level of the control pigs (Table 2). In contrast to abdominal SAT, in VAT none of the proinflammatory cytokines was observed to be affected by cloning.

In the neck SAT (Fig. 1D), cloning affected gene expression very differently than the two other adipose tissues in that four out of the five significantly differentially expressed genes were upregulated; *IL10*, inter-α-trypsin inhibitor heavy-chain family, member 4 (*ITIH4*), acyloxyacyl hydrolase (*AOAH*), and nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor α (*NFKBIA*), whereas only one was downregulated, namely CD36. Furthermore, none of the genes that showed significant expression differences in the adipose tissues from the abdominal region (VAT and abdominal SAT) were significantly regulated in the neck SAT.

Thus, the general trend for the effect of cloning on gene expression was similar for neck SAT and liver in that the affected genes were generally upregulated in the clones. This is strikingly different in VAT and abdominal SAT, both of which reacted quite similarly to cloning with downregulation of gene expression, with the notable exception of the proinflammatory cytokine *TNF*, which was upregulated in abdominal SAT but not VAT of cloned pigs.

These results indicate subtle but distinct and tissue-specific gene expression changes in four different tissues upon cloning. Among the innate immune response genes selected for study here, it was remarkable that APPs were among the upregulated genes in liver tissue, whereas cytokine expression was not affected in this organ. In contrast, APP expression was either unchanged or downregulated in abdominal SAT and VAT.

To investigate whether cloning results in less interindividual variation, we used the F-test for equal variances of all genes in cloned and control pigs. The variance of the two groups was found to be equal ($p=0.08$ for liver, $p=0.66$ for abdominal SAT, $p=0.89$ for VAT, and $p=0.65$ for neck SAT).

Protein concentrations in serum

To analyze if the altered hepatic expression of APPs and cytokine genes could be detected as changes in circulating concentration of the corresponding proteins, the serum concentrations of IL-6, CRP, HP, ORM, SAA, and TNF-α were determined by ELISA (Fig. 2). After removal of possible outlier data points (one for HP and ORM, and two for CRP), no significant differences were found between the cloned and control groups for any of these proteins, although there was a slight (not significant) tendency for HP to be increased and for ORM to be decreased in the cloned group. The variance of the two groups was found to be equal for HP ($p=0.62$) and CRP ($p=0.71$); however, there was a significant difference between the two groups for ORM ($p=0.0002$).

Discussion

Based on optimized RNA extraction methods and employing strict procedures for quality control and validation, we used a chip-based, high-throughput qPCR to obtain accurate measures of the relative expression of innate immune system–related genes in three types of adipose tissue as well as in liver tissue in cloned pigs ($n=8$) compared to normal control pigs ($n=9$).

In accordance with other studies reporting on interindividual variability in pigs cloned by SCNT (Clausen et al., 2011; Hwang et al., 2009; Park et al., 2011; Whyte et al., 2011), we were not able to show significant differences between clones and controls in the interindividual variability in expression of 40 immune system–related genes in the four different tissues. The variability of serum concentrations of two of three serum proteins investigated was also similar in clones and controls, whereas the last serum protein (ORM) was significantly less variable in cloned pigs.

Furthermore, we found subtle changes in the mRNA expression of several innate immune response genes in liver and three types of adipose tissue in the clones compared to the normal outbred pigs. It is assumed that the minimal genetic difference between clones and controls (65% Danish Landrace:35% Yorkshire in clones vs. 75%:25% in controls) is of minor importance.

Expression of innate immune response genes in the liver was largely unaffected by cloning, with the notable exception of genes for APPs, two of which were significantly upregulated to more than 150% of the normal control group, namely *SAA* and *ITIH4*. Borderline upregulated genes in the liver were also mostly APPs (*CRP*, *HP*, and *LBP*), but also included the proinflammatory cytokine *TNF* and the antiinflammatory cytokine *IL10*. However, other proinflammatory mediators like *IL1B* and *IL8* were not affected.

Thus, hepatic APP gene expression might have been directly and selectively affected by the cloning process. Alternatively, the small-scale induction of APP genes seen here may represent a normal physiological response, induced by proinflammatory cytokines not originating from the liver, because the expression of APPs in the liver is affected by disturbance of tissues wherever it may occur (Skovgaard et al., 2009). It is possible that these extrahepatic cytokines could be delivered from the VAT and omental fat via the portal vein as these adipose tissues are drained by the portal circulation. Fontana and co-workers (2007) have found that these tissues affect the liver via endocrine actions in abdominal obesity.

However, *TNF* was the only proinflammatory cytokine that showed a significantly different expression in the clones in either abdominal SAT and/or VAT; *TNF* was upregulated in the abdominal SAT (see below). TNF-α from visceral adipose tissues has been proposed to act locally only (Fontana et al., 2007), so this would support the conclusion that the altered gene expression in the liver seen in the present study may be a direct consequence of cloning. The possibility that other extrahepatic cytokines could affect liver APP expression cannot be ruled out. However, because proinflammatory cytokines like *IL6* and *IL1B* were borderline upregulated in the abdominal SAT (see below), this does not seem likely.

Compared to the liver, VAT and abdominal SAT gene expression was affected very differently by cloning. Although APPs were still the main affected group, they were

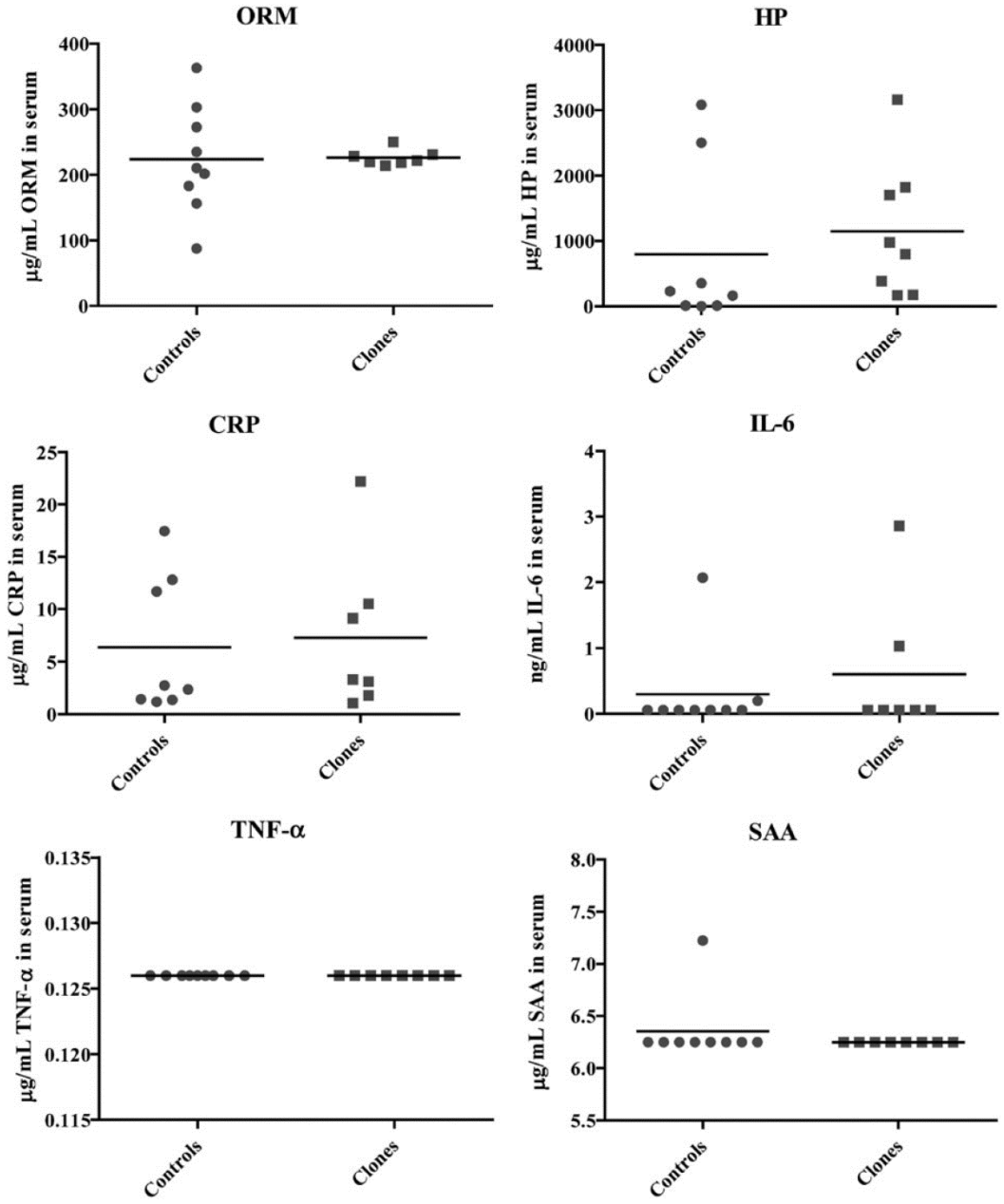

FIG. 2. Scatter plot of serum concentrations of ORM, HP, CRP, IL-6, TNF-α, and SAA measured with ELISA in controls ($n=8$, for ORM $n=9$) and clones ($n=7$, for HP $n=8$). Line indicates mean.

downregulated in both of these tissues, as seen by significant decreases in expression of *SAA*, *HP*, and *C3* in abdominal SAT and *SAA* and *C3* in VAT. Interestingly, this was accompanied by an upregulation of the expression of a subset of proinflammatory cytokines in abdominal SAT (*TNF* significantly and *IL1B* and *IL6* borderline above 150%), whereas expression of the same cytokine subset was not changed or was even downregulated in VAT. Proinflammatory cytokines like IL-1β, IL-6, and TNF-α are extremely potent and have repeatedly been shown to be active both in endo- and paracrine fashion (Vilcek and Le, 1991), and it is remarkable how expression of these cytokines can occur in abdominal SAT without a concomitant induction of APP expression. This could be due to the existence of some mechanism for specific inhibition or

downregulation of the local tissue APP induction in abdominal SAT. Again, however, this seemingly defective APP response may also be a direct consequence of the cloning process itself. Or, possibly, the differences in the expression of these cytokines were not large enough to induce APP expression. Although a defective or downregulated APP expression was also seen in VAT, there was no indication of any effect on proinflammatory cytokine gene expression in this tissue.

Whereas the three above-mentioned types of tissue were found to be affected by cloning with respect to many of the same genes, neck SAT showed a more mixed reaction to cloning, however, with most affected genes being upregulated, placing this tissue alongside the liver with regard to how gene expression was affected by cloning rather than with the other two adipose tissue types.

It should be noted that the expression of the APPs *ORM1*, and *FIB* could not be quantified because the sample dilution used in the assay for all genes was not sufficient to allow quantification of these two genes in all three types of adipose tissue, indicating a very high expression of *ORM1* and *FIB* in these tissues.

None of the differences in APP and cytokine gene expression resulted in significant changes in the serum concentrations of APPs and cytokine in clones compared to controls (Fig. 2). Thus, the subtle changes in expression of mRNA coding for APPs and proinflammatory cytokines found in the tissues investigated do not give rise to discernible systemic effects. This could be due to the combined effect of opposite regulation in more than one endocrine organ, an adaptation of the protein secretion, and/or production pathways to increased gene expression or selective secretion from the tissue in question to the circulation.

The general conclusion of this work is that cloning does not affect gene expression to a large extent in the liver and in the three adipose tissues investigated in 9-month-old pigs; however, small changes were indeed consistently observed in cytokine and APP expression. Even if the effects may seem small compared to those observed during a full-blown acute-phase response to infection (see, for example, Skovgaard et al., 2009, 2010), such small changes may easily result in long-term phenotypic effects. Investigations in human healthy adults with prolonged acute-phase response in the high-normal range have been shown to have an increased long-term risk of angina, myocardial infarction, cardiovascular disease, and death (Munford, 2001; Ridker et al., 2000). It should be noted, however, that although the changes in gene expression observed here are indeed significantly associated with the cloned pigs, it remains to be seen if they are temporary or persist throughout the life of a cloned pig. On the other hand, the fact that a notable number of genes related to inflammatory responses are affected by cloning may have consequences for the usability of cloned pigs as obesity models for the study of the involvement of inflammation in development of obesity-related disease. Whether gene expression of innate immune response genes is different in obese clones and controls is currently being investigated and will be the subject of a subsequent paper.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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Address correspondence to:
Professor Peter M.H. Heegaard
Innate Immunology Group
National Veterinary Institute
Technical University of Denmark
27, Bülowsvej
1870 Frederiksberg C, Denmark

E-mail: pmhh@vet.dtu.dk

3.3.4 Paper II

Cloning changes the response to obesity of innate immune factors in blood, liver and adipose tissues in domestic pigsRødgaard T, Skovgaard K, Stagsted J, Heegaard PMH. Cellular Reprogramming. 2012, submitted as original paper

The layout of the article is changed compared to the submitted version.

The object of this study was to evaluate the usability of cloned pigs as nutritional models. Hopefully, cloned pigs should improve the current porcine nutritional model, as fewer animals will be needed to retain statistical power due to lower inter-individual variation, and the pigs should exhibit a similar immune response as controls. The gene expression of 36 genes in the liver and 31 genes for the three adipose tissues; abdominal SAT, VAT and neck SAT were investigated with high-throughput chip-based qPCR (Fluidigm). Furthermore, the blood serum expression levels of six immune factors were investigated with ELISA (haptoglobin, SAA, ORM1, CRP, IL-6 and TNF- α). This was measured in obese clones and controls, and lean clones and controls. The obese pigs were fed a high fat/high sucrose-diet *ad libitum*, and the lean pigs were fed the same diet, though restricted to 60% of *ad libitum*-diet.

For genes being differentially expressed between the adipose tissues, obesity and cloning decreased the number of differentially expressed genes. These effects were additive so obese, cloned pigs showed little or no genes being differentially expressed between liver and adipose tissues

In the serum no changes in concentrations in any of the six immune factors were detected between the lean and obese controls. However, ORM levels were significantly elevated in the obese clones when compared to the lean clones. Furthermore, two proteins were significantly elevated in the obese clones when compared to the controls, namely ORM and haptoglobin. This suggests that the obese clones are exhibiting a response to the diet that neither the lean clones nor the obese controls are exhibiting.

The variance between the obese clones and controls were tested, and contrary to the study of the lean pigs, a different variance was found between the obese clones and controls in serum proteins ORM and CRP. The inter-individual variation in ORM expression was smaller in the clones, but for CRP it was smaller in the controls.

In conclusion, clear differences between clones and controls, both lean and obese, were found in expression levels of mRNA in tissues and serum expression levels in the blood. As even small changes in the expression of innate immune factors can have detrimental effects in obesity long term, this adds another blow to the benefits of cloned animals as nutritional models.

Cloning changes the response to obesity of innate immune factors in blood, liver and adipose tissues in domestic pigs

Running head: Cloning changes response to obesity in pigs

Tina Rødgaard(1), Kerstin Skovgaard(1), Jan Stagsted(2), Peter M. H. Heegaard(1)

1: Innate Immunology Group, National Veterinary Institute, Technical University of Denmark, Frederiksberg, Denmark

2: Department of Food Science, Faculty of Science and Technology, Aarhus University, Tjele, Denmark

Corresponding author: Professor Peter M. H. Heegaard, Innate Immunology Group, National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, 1870 Frederiksberg C, Denmark. E-mail: pmhh@vet.dtu.dk. Telephone no: +45 35886241. Fax no: + 45 35886001.

Abstract

The objective of the study was to evaluate the usefulness of cloned pigs as porcine obesity models reflecting obesity associated changes in innate immune factor gene expression profiles.

Liver and adipose tissue expression of 43 innate immune genes as well as serum concentrations of six immune factors were analyzed in lean and diet-induced obese cloned domestic pigs compared to normal domestic pigs (obese and lean). The number of genes affected by obesity was lower in cloned animals than in control animals. All genes affected by obesity in adipose tissues of clones were down-regulated; in the controls both up- and down-regulation was observed. Cloning resulted in a less differentiated adipose tissue expression pattern. Finally, the serum concentrations of two acute phase proteins (APPs) HP and ORM were increased in obese clones as compared to obese controls as well as lean clones and controls. Generally, phenotypic variation was not reduced in cloned pigs.

Therefore, we conclude that cloning limits both the number of genes responding to obesity as well as the degree of tissue differentiated gene expression, concomitantly with an increase in APP serum concentrations only seen in cloned, obese pigs. This may suggest that the APP response seen in obese, cloned pigs is an abnormal consequence of the clone specific, skewed gene response to obesity described in this work. This should be taken into consideration when using cloned animals as models for innate responses to obesity.

Introduction

Obesity is associated with a chronic activation of the innate immune system resulting in a low-grade inflammation of the white adipose tissue, which has been implicated in the development of obesity related diseases such as type 2 diabetes, cardiovascular diseases and hepatic steatosis constituting important parts of the metabolic syndrome (Bastard et al., 2006;Federico et al., 2010;Gil et al.,

2007). Soluble mediators of the innate immune system include proteins such as cytokines, chemokines and acute phase proteins (APPs). These proteins are mainly produced by cells of the immune system and by the liver, however, several of these proteins have been shown to be produced by the adipose tissues as well, such as C-reactive protein (CRP), IL-6 and TNF-α (reviewed in (Fain, 2010;Fantuzzi, 2005)). Low-grade inflammation in the adipose tissues is not only a local phenomenon as elevated serum levels of pro-inflammatory cytokines and APPs have been found in obese individuals (Chiellini et al., 2004;Mendall et al., 1997) and Mohamed-Ali and coworkers (Mohamed-Ali et al., 1997) found that 30% of the circulating interleukin-6 (IL-6) is derived from adipocytes in obese individuals. Furthermore, serum concentrations of APPs can be used as a predictor of weight gain (Engström et al., 2003).

Pigs are promising models for the obesity-induced inflammation-related responses thought to be the underlying cause of the metabolic syndrome (Litten-Brown et al., 2010). Cloned pigs should in theory show less inter-individual variation as they have identical genotypes and therefore are expected to have more uniform phenotypes than normal outbred pigs and thus have potential to improve the model. However, it has been shown by us and others that inter-individual phenotypic variation was not reduced for a number of traits by cloning, including expression levels of specific genes (Rødgaard et al., 2012;Tian et al., 2008). Even though several reports have shown that animals cloned by somatic cell nuclear transfer (SCNT) are healthy and normal, other investigations report deviant phenotypes (Clausen et al., 2011;Hwang et al., 2009;Park et al., 2011;Whyte et al., 2011), including lower body weights (Park et al., 2005;Tian et al., 2008). Furthermore, cloning by SCNT leads to higher perinatal and early postnatal mortality (Park et al., 2005;Whitworth et al., 2009). This has been attributed to a large number of causes, including metabolic and cardiopulmonary abnormalities (Hill et al., 1999;Wells et al., 1999), lymphoid hypoplasia (Renard et al., 1999) and neonatal respiratory distress (Hill et al., 1999), but also, interestingly, to bacterial

infections (Carter et al., 2002;Keefer et al., 2001;Peura et al., 2003). Studies in cloned piglets (Carter et al., 2002) and cloned cattle (Chavatte-Palmer et al., 2009) have revealed that the adaptive immune system is apparently not affected by the cloning process. However, we have previously described that basal innate immune response gene expression is altered in cloned pigs compared to normal outbred pigs (Rødgaard et al., 2012) supported by earlier findings by Carroll and coworkers (Carroll et al., 2005) on altered innate immune responses to lipopolysaccharide in cloned pigs. All of this has raised concerns regarding the use of cloned animals in metabolic and knock-out analysis (St John et al., 2005) and there is a need for further investigations into the use of cloned animals in nutrition studies to supplement the few studies which have already been conducted (Christensen et al., 2012;Jensen et al., 2010).

Therefore the objective of this study was to evaluate the usability of cloned pigs in nutritional studies by looking at the expression of innate immune genes and serum levels of APPs and cytokines in obese cloned pigs and to investigate whether cloning reduced inter-individual variation in these parameters and whether cloning had any other effects on these parameters. Cloned pigs (n=9) and normal outbred pigs (controls, n=10) fed a high fat/high sucrose diet ad libitum (obese group), and cloned pigs (n=8) and normal outbred pigs (controls, n=9) fed a high fat/high sucrose diet restricted to 60% of ad libitum intake (lean group) were compared.

Materials and procedures

All methods and descriptions of the lean clones and controls have been previously described in (Rødgaard et al., 2012). The following is a brief summary.

Animals, diets and sampling

All experimental procedures involving animals were approved by the Danish Animal Experimental Committee.

Somatic cell nuclear transfer was used to perform the cloning, as described in (Kragh et al., 2004). Donor cells were from cultured ear fibroblasts obtained from a Danish Landrace x Yorkshire (65%:35%) sow and the cloned embryos were transferred to surrogate sows five to six days after cloning, as described in (Schmidt et al., 2010). Controls were normal litters (either 36%:64% or 75%:25%) obtained by standard insemination. The lean pigs are described in (Rødgaard et al., 2012).

The obese pigs were from two groups born a year apart. Group 1 consisted of four clones and four controls, and group 2 consisted of five clones and six controls. Piglets from groups 1 were delivered normally and piglets from group 2 were delivered by Ceasarian section on gestation day 116 (Schmidt et al., 2010), with treatment with a prostaglandin analogue (175 µg Estrumate i.m., Pitman-Morre, UK) 24 h before Ceasarian section. All pigs were reared in the experimental stables of Aarhus University (Tjele, Denmark). Pigs were weaned after 28 days and at three months of age the pigs were fed a high-energy diet (containing 10% sugar and 10% soy oil) ad libitum (the diet is described in (Christensen et al., 2012)). At an age of 7-8 months the pigs were killed with a bolt pistol after overnight fasting. The animals were desangiunated and the carcass split into two halves; the right side was used for tissue sampling and the left side was used for CT scanning. Tissue samples were snap frozen in liquid nitrogen. Blood was allowed to clot at room temperature for 1 hour, centrifuged at 3000 rpm for 10 minutes at 4°C and aliquots of serum were then frozen. Tissue and serum samples were kept at -80°C until analysis. CT scanning was performed with a High-speed single-slice CTi instrument from General Electric, obtaining slices of 5 mm resolution. CT data were analyzed using VG MAX 2.1 software (Volume Graphics GmBH, Germany) for quantification of fat and muscle volume.

Extraction of RNA

For RNA extracted from abdominal fat samples from group 1 and liver tissue from group 1 and 2 the following method was used: Total RNA from ~100 mg of liver tissue was isolated by a RNeasy Lipid tissue Midi kit (Qiagen, Ballerup, Denmark) and ~1 g of abdominal fat tissue was isolated by a RNeasy Maxi kit (Qiagen) and QIAzol Lysis Reagent (Qiagen) according to manufacturer's protocol. Both were treated with on-column RNase-free DNase digestion (Qiagen). The tissues from group 1 were homogenized with a hand blender, where the liver tissue from group 2 was homogenized on the gentleMACS Dissociator (Miltenyi Biotec, Germany) in gentleMACS M tubes (Miltenyi Biotec).

For the abdominal SAT from group 2 as well as the VAT and neck SAT from group 1 and 2 the method of RNA extraction with phenol and chloroform was used. ~1 g of fatty tissue was homogenized with QIAzol Lysis Reagent on the gentleMACS Dissociator after which chloroform was added. After centrifugation isopropanol (2-propanol) was added to the upper, aqueous phase. The tube was centrifuged and the pellet was washed 3 times in cold 75% EtOH. The supernatant was removed completely and pellet was left to air dry. The RNA was dissolved in RNase-free water. The RNA yield was then measured on the NanoDrop (ND-1000 Spectrophotometer, NanoDrop Technologies Inc, USA).

RNA integrity

For the assessment of RNA integrity the Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) was used. The RNA integrity number (RIN) was identified with the use of Agilent RNA 6000 Nano Kit (Agilent Technologies) according to manufacturer's protocol. In the liver all samples had a RIN of above 8, whereas all samples in the fatty tissues had a RIN of above 6.2.

cDNA synthesis and pre amplification

Each sample of extracted RNA was run in duplicates and 500 ng total RNA was reverse transcribed using the QuantiTECT Reverse Transcription kit (Qiagen), according to the manufacturer's instructions. Prior to pre amplification, which was performed using TaqMan PreAmp Master Mix (Applied Biosystems, CA, USA), the cDNA was diluted in low EDTA TE-buffer (VWR – Bie & Berntsen, Herlev, Denmark). Pre amplified cDNA was diluted in low EDTA TE-buffer before qPCR.

Primer design and validation

Primers were designed using Primer3 (http://frodo.wi.mit.edu/) as described previously in (Skovgaard et al., 2009) and synthesized at TAG Copenhagen (Copenhagen, Denmark). Primer specificities, primer amplification efficiencies, dynamic ranges, specificity and sequences was tested and described in (Rødgaard et al., 2012).

Quantitative real time PCR (qPCR)

qPCR was performed in 48.48 Dynamic Array Integrated Fluidic Circuits (Fluidigm, CA, USA), combining 48 samples with 48 primer sets for 2304 simultaneous qPCR reactions in the BioMark real-time RCR instrument (FluiDigm). Reaction mix was prepared with ABI TaqMan Gene Expression Master Mix (Applied Biosystems), 20X DNA Binding Dye Sample Loading Reagent (Fluidigm), 20X EvaGreen (Biotium, VWR – Bie & Berntsen), and low EDTA TE Buffer. Reaction mix was mixed with pre amplified cDNA, diluted in low EDTA TE-buffer. Specific primer mix were prepared using 20 µM primer (forward and reverse), 2X Assay Loading Reagent (Fluidigm) and low EDTA TE-buffer.

Expression data were acquired using the Fluidigm Real-Time PCR Analysis software 3.0.2 (Fluidigm) and exported to GenEx5 ((MultiD, Göteborg, Sweden).

Data analysis and statistics

GenEx5 was used for data pre-processing, normalisation, relative quantification and statistics. Data were corrected for PCR efficiency for each primer assay individually. The most stable expressed reference genes were found for each tissue type individually as well as for all samples combined out of a panel of five putative reference genes using GeNorm (Vandesompele et al., 2002) and the geometric mean of these were used for normalization in GenEx5. Expressions were calculated relative to the least expressed samples for each primer assay and data were log2 transformed to approach normal distribution prior to two-tailed, unpaired t-test, one-way ANOVA with Tukey-Kramer post test, and/or F-test. Gene expression changes were considered to be significant if $p \leq 0.05$ with a fold change of at least ±1.5. Data are expressed as the mean ± standard error of the mean (SEM).

Quantitative serum ELISA

The protein concentrations in serum of CRP, HP, ORM, IL-6, TNF-α and SAA were analyzed by sandwich ELISAs. CRP, HP and ORM was analyzed with in-house assays, where commercially available ELISA assays were used to determine IL-6 (Porcine IL-6 Duoset kit, R&D Systems, MN, USA), TNF-α (Swine TNF-α CytoSetTM, Invitrogen,CA, USA, with Antibody Pair Buffer Kit (Invitrogen, CA, USA)) and SAA (Phase SAA assay, Tridelta Development Ltd., Kildare, Ireland). All ELISAs were developed using TMB-plus (KEM-EN-TEC, Taastrup, Denmark) according to the manufacturer's instructions and read using an automatic plate reader (Thermo Multiskan Ex spectrophotometer, Thermo Scientific, Waltham, MA, USA). Within a single assay all samples

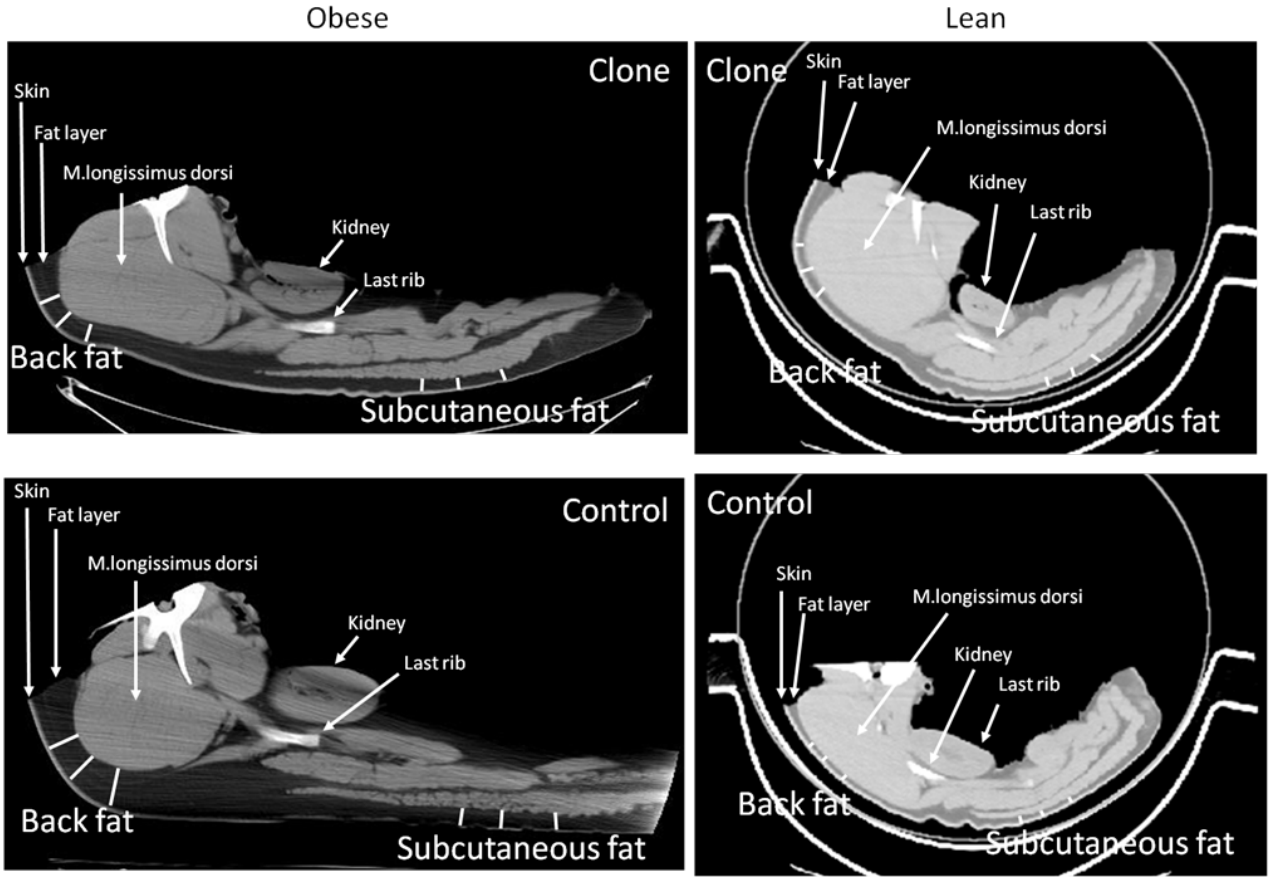
were run in duplicates and an intra-assay coefficient of variation of <15% was accepted. Ascent software v. 2.6, Thermo Scientific was used to calculate sample values and significance was tested using a two-tailed unpaired t-test where variance was tested with an F-test. $p \leq 0.05$ was considered significant. Outliers were removed from dataset after evaluation with Grubbs outlier test (Grubbs, 1969) where applicable. Data are expressed as the mean ± standard error of the mean (SEM).

Results

Pigs

When comparing the four groups (lean controls, lean clones, obese controls and obese clones) at the time of slaughter there was no significant difference in the weight of the lean controls and lean clones (Rødgaard et al., 2012). The weights of the obese clones and controls were significantly higher than the lean clones ($p<0.0001$) and lean controls ($p=0.03$), respectively. However, the obese control animals weighed significantly more than the obese clones (170.1±4.9 kg and 147.5±5.9 kg, respectively, $p=0.009$). CT scans were performed on all lean pigs and on 6 obese controls and 5 obese clones, selected to be representative of the groups. A representative CT scan from each group is shown in Figure 1 and the percentage of total body fat calculated from these scans is shown in Figure 2. The CT scans showed a significantly higher percentage of total body fat ($p=0.002$) in the obese controls when compared to the obese clones. Furthermore, the obese controls had more back fat ($p<0.0001$) and abdominal SAT ($p<0.0001$) than the obese clones (data not shown). In addition, a significantly higher proportion of total body fat was seen in the obese controls (40.1%±1.5%) and obese clones (28.4%±2.3%) compared to the lean controls (21.8%±1.3%, $p<0.0001$) and lean clones (18.4%±2.5%, $p=0.02$), respectively.

Figure 1: Images from CT scan



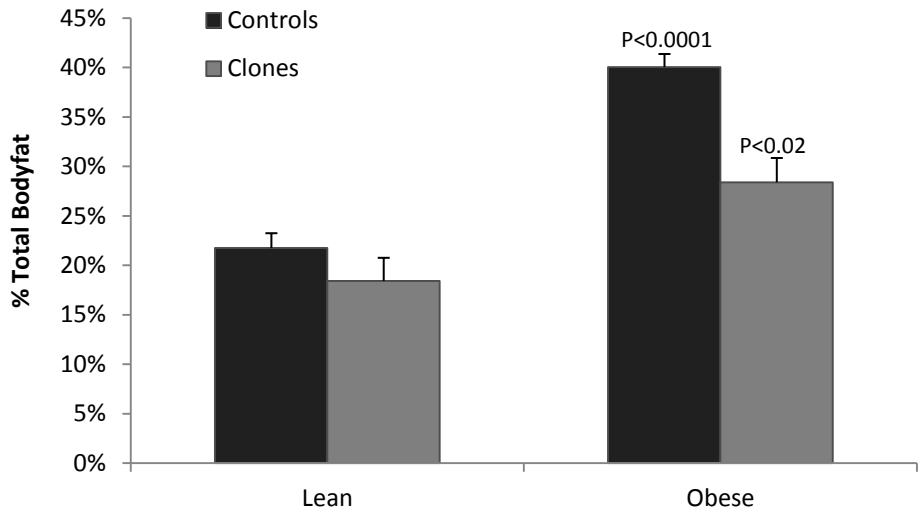
Representative CT scans of obese controls (A) and clones (B) and lean controls (C) and clones (D) done at endpoint. The thickness of fat was measured at the 3 lines drawn in the back fat and abdominal SAT, and a mean of the three measurements were found. Arrows indicate ribs, kidney, fat, skin and muscle. All measurements were done in scans from the last rib.

Gene expression

A total of 43 immune related genes and 5 reference genes were analyzed. After quality control as described in (Rødgaard et al., 2012), a number of genes were found to yield sub-quality data and were excluded from further evaluation. This included MUC1, DEFB1 and IL12 for all tissues, SFTPA1, FIB, IFNG, LBP, IL1RN, ORM1 and IL10(b) for all adipose tissues and IL8(a) and IL6 for the liver. Furthermore, when duplicate primer sets were used with similar results, only the result from one of the primer sets is shown (IL8(a) in the adipose tissues, IL10(a) for the liver and ITIH4(b) for all tissues). Significantly differentially expressed genes were defined as genes showing

a statistically significant difference in expression ($p \leq 0.05$) and a fold change of at least ±1.5, comparing either two pig groups or two types of tissues (tissue expression patterns).

Figure 2: Percent total body fat



Percentage of total body fat in lean and obese clones and controls, as measured from CT scans done at endpoint. n=9 for lean controls, n=8 for lean clones, n=6 for obese controls, and n=5 for obese clones. Error bars depict SEM.

The expression data for obese clones and obese controls (set to 1), for all tissues, are shown in Supplementary Table 1, while Table 1 shows expression levels in adipose tissues (abdominal SAT and VAT) relative to neck SAT (set to 1) for all four pig groups, illustrating the tissue expression patterns for the 31 genes.

Figure 3 shows the expression for all four tissues investigated of all genes that were significantly differentially expressed in obese as compared to lean pigs (set to 1) for both controls and for clones.

Effect of cloning

As a general observation, the inter-individual variability in gene expression between pigs in the group of obese clones was not different from that observed between individual pigs of the obese

control group as analyzed by the F-test (p=0.54 for liver, p=0.36 for abdominal SAT, p=0.87 for VAT and p=0.72 for neck SAT).

Only few genes showed a significantly changed expression in obese clones compared to obese controls (Supplementary Table 1). Two genes were slightly (LBP) or moderately (SAA) significantly up-regulated in the liver, whereas one gene in each of the adipose tissues was affected by cloning (IL1B in abdominal SAT, C3 in VAT and IL18 in neck SAT).

Tissue specific expression patterns, i.e. the relative expression in the three adipose tissues using the level of expression in neck SAT as a reference (i.e. set to 1 for each group) were compared for 31 genes in obese clones versus obese controls (Table 1). In the obese control group, differential expression was seen for a substantial number of genes with five genes being higher expressed in abdominal SAT than in neck SAT and five genes being higher expressed and one lower expressed in VAT than in neck SAT. In contrast, in the obese clones, only two genes in abdominal SAT and one gene in VAT were differentially expressed compared to neck SAT, all being higher expressed. In comparison, in the lean control group nine genes in abdominal SAT and 13 in VAT were significantly differentially expressed compared to neck SAT (all except two genes were higher expressed), while the corresponding numbers for lean clones were two and three, respectively. Differential expression in abdominal SAT compared to VAT (Table 1) were seen for eight genes in obese controls and for no genes in obese clones. In the lean group six genes were differentially expressed in abdominal SAT compared to VAT in the controls and two in the clones.

Thus, both cloned groups showed a much reduced differentiated adipose tissue specific gene expression.

Supplementary Table 1: Overview of gene expression in the liver and three adipose tissues

	Liver		Abdominal SAT		VAT		Neck SAT	
	Controls	Clones	Controls	Clones	Controls	Clones	Controls	Clones
AOAH	1.00 ± 0.11	1.35 ± 0.14	1.00 ± 0.21	0.91 ± 0.10	1.00 ± 0.15	1.26 ± 0.16	1.00 ± 0.33	0.84 ± 0.21
APOA1	1.00 ± 0.08	1.10 ± 0.11	1.00 ± 0.15	0.86 ± 0.08	1.00 ± 0.25	1.01 ± 0.17	1.00 ± 0.08	0.92 ± 0.09
C3	1.00 ± 0.06	1.00 ± 0.13	1.00 ± 0.32	0.49 ± 0.12	1.00 ± 0.15	0.51 ± 0.13a	1.00 ± 0.12	1.33 ± 0.32
CCL2	1.00 ± 0.28	0.85 ± 0.22	1.00 ± 0.12	1.02 ± 0.11	1.00 ± 0.25	0.86 ± 0.16	1.00 ± 0.09	0.86 ± 0.07
CCL3L1	1.00 ± 0.17	1.13 ± 0.21	1.00 ± 0.11	0.72 ± 0.06	1.00 ± 0.32	1.32 ± 0.44	1.00 ± 0.29	0.65 ± 0.09
CCL5	1.00 ± 0.11	1.15 ± 0.09	1.00 ± 0.08	1.27 ± 0.16	1.00 ± 0.22	0.98 ± 0.12	1.00 ± 0.07	1.03 ± 0.17
CD14	1.00 ± 0.07	1.09 ± 0.09	1.00 ± 0.10	0.99 ± 0.12	1.00 ± 0.23	1.31 ± 0.19	1.00 ± 0.05	0.89 ± 0.07
CD163	1.00 ± 0.11	1.34 ± 0.10	1.00 ± 0.13	1.12 ± 0.18	1.00 ± 0.23	1.11 ± 0.18	1.00 ± 0.09	0.91 ± 0.12
CD200	1.00 ± 0.09	0.99 ± 0.10	1.00 ± 0.13	1.18 ± 0.12	1.00 ± 0.13	1.11 ± 0.10	1.00 ± 0.05	0.81 ± 0.08
CD36	1.00 ± 0.09	1.11 ± 0.12	1.00 ± 0.10	0.93 ± 0.12	1.00 ± 0.12	1.27 ± 0.16	1.00 ± 0.05	1.01 ± 0.05
CD40	1.00 ± 0.07	0.97 ± 0.08	1.00 ± 0.12	1.01 ± 0.11	1.00 ± 0.28	1.03 ± 0.33	1.00 ± 0.04	0.87 ± 0.08
COX-2	1.00 ± 0.37	1.22 ± 0.38	1.00 ± 0.16	1.01 ± 0.22	1.00 ± 0.30	0.62 ± 0.24	1.00 ± 0.32	0.68 ± 0.15
CRP	1.00 ± 0.21	2.11 ± 0.54	1.00 ± 0.59	0.12 ± 0.06	1.00 ± 0.13	1.21 ± 0.21	1.00 ± 0.09	1.61 ± 0.60
CXCL10	1.00 ± 0.18	0.71 ± 0.06	1.00 ± 0.11	0.88 ± 0.11	1.00 ± 0.22	1.20 ± 0.24	1.00 ± 0.19	0.70 ± 0.06
FIB	1.00 ± 0.11	1.12 ± 0.14	NQ	NQ	NQ	NQ	NQ	NQ
HP	1.00 ± 0.07	1.24 ± 0.15	1.00 ± 0.60	0.09 ± 0.06	1.00 ± 0.26	0.58 ± 0.13	1.00 ± 0.15	3.15 ± 1.53
IFNG	1.00 ± 0.22	1.46 ± 0.36	NQ	NQ	NQ	NQ	NQ	NQ
IL10	1.00 ± 0.14	1.65 ± 0.36	1.00 ± 0.14	0.95 ± 0.10	1.00 ± 0.17	1.05 ± 0.15	1.00 ± 0.06	0.84 ± 0.11
IL18	1.00 ± 0.08	1.34 ± 0.07	1.00 ± 0.24	1.66 ± 0.36	1.00 ± 0.23	1.35 ± 0.41	1.00 ± 0.04	1.59 ± 0.27a
IL1B	1.00 ± 0.13	1.25 ± 0.24	1.00 ± 0.18	1.97 ± 0.35a	1.00 ± 0.54	1.97 ± 1.04	1.00 ± 0.20	1.77 ± 0.42
IL6	NQ	NQ	1.00 ± 0.14	0.98 ± 0.15	1.00 ± 0.44	1.48 ± 0.60	1.00 ± 0.18	0.81 ± 0.10
IL1RN	1.00 ± 0.14	0.77 ± 0.11	NQ	NQ	NQ	NQ	NQ	NQ
IL8(a)	1.00 ± 0.29	0.57 ± 0.09	1.00 ± 0.43	1.02 ± 0.28	1.00 ± 0.48	0.29 ± 0.09	1.00 ± 0.19	1.54 ± 0.34
ITIH4(a)	1.00 ± 0.10	1.67 ± 0.34	1.00 ± 0.58	0.24 ± 0.09	1.00 ± 0.15	0.72 ± 0.12	1.00 ± 0.12	1.11 ± 0.16
LBP	1.00 ± 0.23	1.80 ± 0,31a	NQ	NQ	NQ	NQ	NQ	NQ
NFkB1	1.00 ± 0.06	0.93 ± 0.06	1.00 ± 0.05	1.05 ± 0.05	1.00 ± 0.09	1.05 ± 0.07	1.00 ± 0.03	0.92 ± 0.04
NFKBIA	1.00 ± 0.07	0.91 ± 0.09	1.00 ± 0.05	1.11 ± 0.09	1.00 ± 0.11	0.91 ± 0.11	1.00 ± 0.06	1.03 ± 0.11
ORM1	1.00 ± 0.09	1.25 ± 0.17	NQ	NQ	NQ	NQ	NQ	NQ
PAFAH1B1	1.00 ± 0.06	0.92 ± 0.05	1.00 ± 0.03	1.02 ± 0.04	1.00 ± 0.08	1.03 ± 0.08	1.00 ± 0.02	0.92 ± 0.05
PDB2	1.00 ± 0.19	1.26 ± 0.32	1.00 ± 0.14	0.66 ± 0.11	1.00 ± 0.24	1.15 ± 0.25	1.00 ± 0.07	0.96 ± 0.10
SFTPA1	1.00 ± 0.36	0.59 ± 0.15	NQ	NQ	NQ	NQ	NQ	NQ
SAA	1.00 ± 0.24	3.06 ± 1,17a	1.00 ± 0.66	0.13 ± 0.10	1.00 ± 0.23	0.52 ± 0.04	1.00 ± 0.29	1.33 ± 0.73
TF	1.00 ± 0.08	1.22 ± 0.08	1.00 ± 0.59	0.05 ± 0.01	1.00 ± 0.46	0.83 ± 0.20	1.00 ± 0.28	1.99 ± 0.84
TGFB1	1.00 ± 0.07	1.03 ± 0.07	1.00 ± 0.07	1.06 ± 0.08	1.00 ± 0.15	0.93 ± 0.14	1.00 ± 0.04	1.00 ± 0.10
TLR4	1.00 ± 0.09	1.20 ± 0.10	1.00 ± 0.13	0.98 ± 0.15	1.00 ± 0.14	1.15 ± 0.17	1.00 ± 0.07	0.78 ± 0.05
TNF	1.00 ± 0.13	1.20 ± 0.20	1.00 ± 0.15	1.12 ± 0.11	1.00 ± 0.20	1.33 ± 0.20	1.00 ± 0.14	0.97 ± 0.07
TNFAIP3	1.00 ± 0.09	0.80 ± 0.09	1.00 ± 0.11	0.93 ± 0.09	1.00 ± 0.13	1.31 ± 0.28	1.00 ± 0.08	0.85 ± 0.13

Relative innate immune gene expression in liver, abdominal SAT, VAT and neck SAT in obese cloned pigs as compared to obese controls (set to 1), as analyzed by qPCR ± SEM. a significant difference with a fold change of at least ±1.5; NQ = not quantifiable.

Table 1: Comparative gene expression of the three adipose tissues in lean and obese pigs

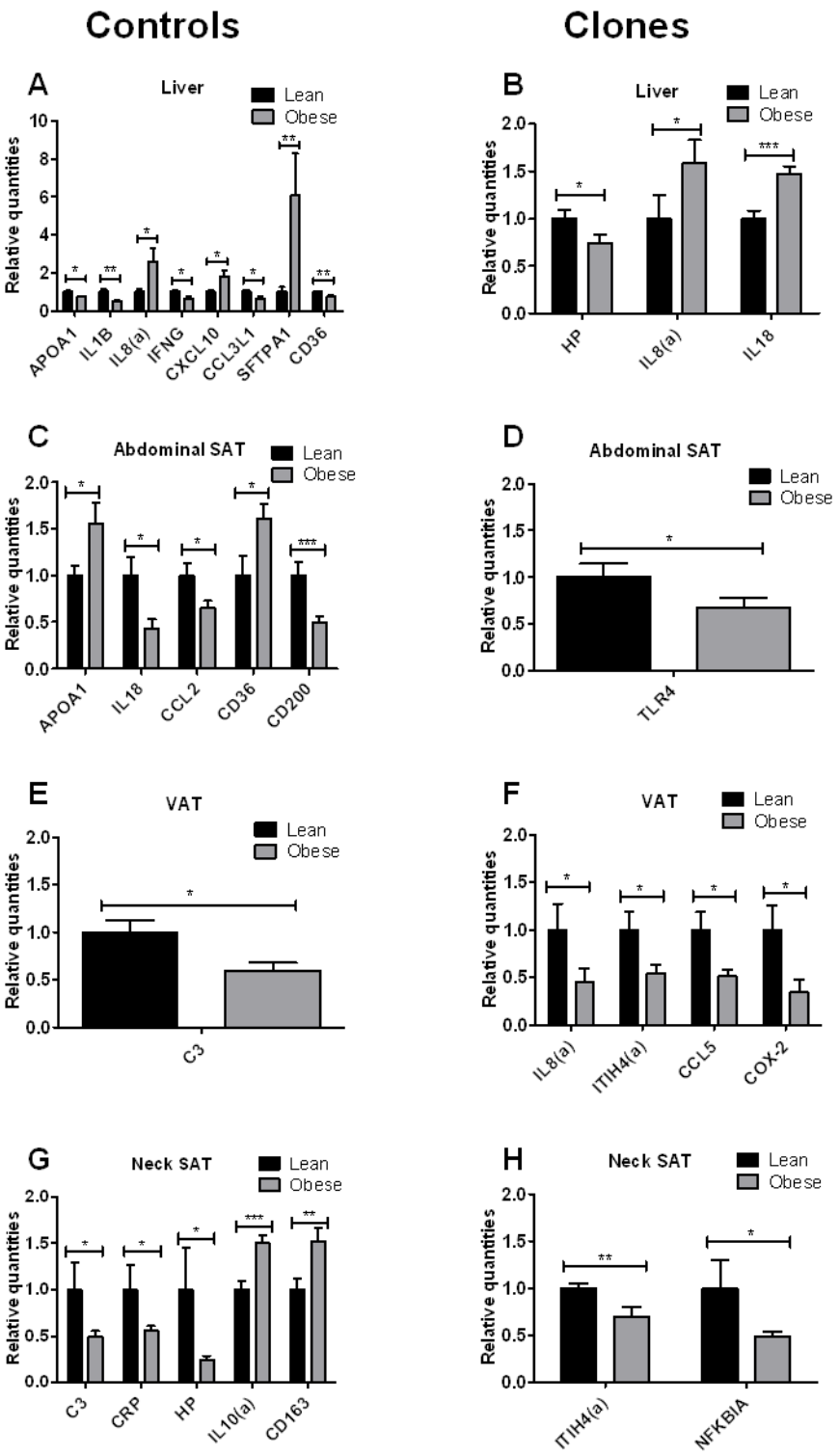
	Lean						Obese					
	Controls (n=9)			Clones (n=8)			Controls (n=10)			Clones (n=9)		
	Abd. SAT	VAT	Neck SAT	Abd. SAT	VAT	Neck SAT	Abd. SAT	VAT	Neck SAT	Abd. SAT	VAT	Neck SAT
APOA1	0.71 ± 0.08	0.70 ± 0.13	1.00 ± 0.05	0.64 ± 0.09	0.72 ± 0.07	1.00 ± 0.14	1.00 ± 0.14	0.68 ± 0.17	1.00 ± 0.08	0.94 ± 0.09	0.76 ± 0.12	1.00 ± 0.05
CD14	1.75 ± 0.19	0.95 ± 0.13	1.00 ± 0.06	1.66 ± 0.21	1.07 ± 0.13	1.00 ± 0.10	1.52 ± 0,15b	0.84 ± 0,19b	1.00 ± 0.05	1.43 ± 0.18	1.04 ± 0.15	1.00 ± 0.06
CD40	2.42 ± 0,30a	3.02 ± 0,98a	1.00 ± 0.10	1.47 ± 0.16	1.49 ± 0.10	1.00 ± 0.09	1.28 ± 0.16	2.05 ± 0.58	1.00 ± 0.04	1.71 ± 0.19	2.78 ± 0.89	1.00 ± 0.10
C3	3.28 ± 0,88a	4.73 ± 0,60a	1.00 ± 0.29	1.42 ± 0.32	2.45 ± 0.53	1.00 ± 0.14	5.75 ± 1,83a	5.68 ± 0,84a	1.00 ± 0.12	1.41 ± 0.33	1.43 ± 0.38	1.00 ± 0.29
CRP	0.79 ± 0.09	0.77 ± 0.34	1.00 ± 0.27	1.16 ± 0.11	0.63 ± 0.05	1.00 ± 0.15	15.68 ± 9,21ab	0.61 ± 0,08b	1.00 ± 0.09	1.08 ± 0.55	0.41 ± 0.07	1.00 ± 0.27
PDB2	1.58 ± 0.33	1.10 ± 0.26	1.00 ± 0.13	0.39 ± 0.06	0.38 ± 0.11	1.00 ± 0.48	1.25 ± 0.17	1.01 ± 0.24	1.00 ± 0.07	0.78 ± 0.12	1.08 ± 0.24	1.00 ± 0.13
HP	1.77 ± 0.83	2.14 ± 1.30	1.00 ± 0.45	0.44 ± 0.17	0.46 ± 0.08	1.00 ± 0.69	32.13 ± 19,24ab	1.98 ± 0,52b	1.00 ± 0.15	0.74 ± 0.47	0.28 ± 0.06	1.00 ± 0.45
IL8(a)	6.78 ± 2,59a	17.01 ± 8,47a	1.00 ± 0.28	0.98 ± 0.22	1.88 ± 0,44a	1.00 ± 0.82	3.09 ± 1.20	10.49 ± 4,72a	1.00 ± 0.14	3.07 ± 0.79	3.49 ± 1.10	1.00 ± 0.28
IL1b	2.83 ± 0.77	11.72 ± 4,43a	1.00 ± 0.20	2.26 ± 0.66	4.54 ± 1.71	1.00 ± 0.38	1.58 ± 0.28	5.63 ± 3,03a	1.00 ± 0.20	2.73 ± 0.48	9.73 ± 5.11	1.00 ± 0.20
IL6	0.47 ± 0,17b	2.83 ± 0,73b	1.00 ± 0.20	0.81 ± 0.29	1.74 ± 0.50	1.00 ± 0.56	1.22 ± 0.17	2.68 ± 1.17	1.00 ± 0.18	0.63 ± 0.10	2.09 ± 0.84	1.00 ± 0.20
IL10(a)	2.43 ± 0,26a	1.95 ± 0,22a	1.00 ± 0.09	1.35 ± 0.18	1.12 ± 0.18	1.00 ± 0.31	1.38 ± 0.19	1.48 ± 0.25	1.00 ± 0.06	1.97 ± 0.21	2.33 ± 0,34a	1.00 ± 0.09
IL18	8.51 ± 1,70a	4.68 ± 1,66a	1.00 ± 0.10	2.66 ± 0,52a	1.52 ± 0.40	1.00 ± 0.48	3.00 ± 0,73a	3.46 ± 0,81a	1.00 ± 0.04	6.05 ± 1,33a	5.68 ± 1.74	1.00 ± 0.10
ITIH4(b)	0.99 ± 0.10	1.69 ± 0.41	1.00 ± 0.07	0.96 ± 0.18	0.99 ± 0.18	1.00 ± 0.05	7.25 ± 4.20	1.21 ± 0.18	1.00 ± 0.12	1.59 ± 0.60	0.81 ± 0.14	1.00 ± 0.07
SAA	32.54 ± 21,62a	1.94 ± 0.29	1.00 ± 0.17	1.88 ± 0.50	0.57 ± 0.06	1.00 ± 0.15	45.37 ± 30,16ab	1.15 ± 0,26b	1.00 ± 0.29	7.45 ± 5,42a	0.74 ± 0.06	1.00 ± 0.17
TGFB	1.75 ± 0,10a	2.09 ± 0,26a	1.00 ± 0.05	1.39 ± 0.09	1.54 ± 0.14	1.00 ± 0.09	1.22 ± 0.09	1.54 ± 0.22	1.00 ± 0.04	1.51 ± 0.12	1.67 ± 0.25	1.00 ± 0.05
TNF	1.25 ± 0.16	1.54 ± 0.36	1.00 ± 0.14	1.23 ± 0.15	0.97 ± 0.16	1.00 ± 0.22	0.98 ± 0.15	1.28 ± 0.25	1.00 ± 0.14	1.50 ± 0.15	2.33 ± 0.35	1.00 ± 0.14
TLR4	1.68 ± 0,27a	0.78 ± 0.08	1.00 ± 0.03	1.85 ± 0,27b	0.87 ± 0,13b	1.00 ± 0.11	1.21 ± 0,15b	0.67 ± 0,10b	1.00 ± 0.07	1.29 ± 0.20	0.84 ± 0.12	1.00 ± 0.03
AOAH	1.22 ± 0.13	1.16 ± 0.20	1.00 ± 0.06	0.95 ± 0.10	0.94 ± 0.09	1.00 ± 0.10	0.92 ± 0.19	0.76 ± 0.11	1.00 ± 0.33	1.52 ± 0.16	1.76 ± 0.22	1.00 ± 0.06
CXCL10	1.79 ± 0.49	2.16 ± 0.46	1.00 ± 0.14	0.60 ± 0.36	1.12 ± 0.87	1.00 ± 0.88	1.30 ± 0.14	1.18 ± 0.26	1.00 ± 0.19	1.28 ± 0.15	1.57 ± 0.31	1.00 ± 0.14
CCL3LI	1.43 ± 0,23b	6.19 ± 2,47ab	1.00 ± 0.20	1.81 ± 0.19	4.85 ± 0,93a	1.00 ± 0.17	1.14 ± 0.13	1.80 ± 0.58	1.00 ± 0.29	1.38 ± 0.12	3.99 ± 1.33	1.00 ± 0.20
CCL2	1.89 ± 0.25	3.66 ± 0,72a	1.00 ± 0.14	1.45 ± 0.15	2.01 ± 0.46	1.00 ± 0.31	1.05 ± 0.13	1.85 ± 0.47	1.00 ± 0.09	1.25 ± 0.14	1.87 ± 0.36	1.00 ± 0.14
CCL5	1.17 ± 0,24b	2.59 ± 0,28ab	1.00 ± 0.12	0.84 ± 0,05b	2.76 ± 0,49ab	1.00 ± 0.10	0.92 ± 0,07b	1.87 ± 0,42b	1.00 ± 0.07	1.26 ± 0.16	2.00 ± 0.24	1.00 ± 0.12
PAFAH1B1	0.99 ± 0,03b	0.71 ± 0,05ab	1.00 ± 0.02	1.14 ± 0.07	0.90 ± 0.06	1.00 ± 0.04	0.90 ± 0.03	0.76 ± 0.06	1.00 ± 0.02	0.90 ± 0.04	0.77 ± 0.06	1.00 ± 0.02
TNFAIP3	0.98 ± 0.08	1.29 ± 0.21	1.00 ± 0.06	1.03 ± 0.12	1.29 ± 0.23	1.00 ± 0.14	1.03 ± 0.11	1.14 ± 0.15	1.00 ± 0.08	0.94 ± 0.09	1.47 ± 0.31	1.00 ± 0.06
CD163	1.93 ± 0.16	1.27 ± 0.15	1.00 ± 0.12	1.54 ± 0.21	0.96 ± 0.12	1.00 ± 0.13	1.37 ± 0.17	1.01 ± 0.23	1.00 ± 0.09	2.34 ± 0.37	1.72 ± 0.29	1.00 ± 0.12
NFKB1	1.27 ± 0.09	1.08 ± 0.07	1.00 ± 0.02	1.11 ± 0.09	0.96 ± 0.06	1.00 ± 0.14	1.09 ± 0.06	0.95 ± 0.08	1.00 ± 0.03	1.14 ± 0.06	0.99 ± 0.07	1.00 ± 0.02
NFKBIA	1.02 ± 0,12b	1.94 ± 0,30ab	1.00 ± 0.08	0.63 ± 0.08	0.90 ± 0.10	1.00 ± 0.30	0.98 ± 0,05b	1.52 ± 0,17ab	1.00 ± 0.06	1.07 ± 0.09	1.36 ± 0.16	1.00 ± 0.08
TF	1.67 ± 1.11	1.86 ± 1.47	1.00 ± 0.34	0.53 ± 0.28	0.13 ± 0.03	1.00 ± 0.69	11.62 ± 6,80b	0.38 ± 0,18b	1.00 ± 0.28	0.26 ± 0.06	0.14 ± 0.03	1.00 ± 0.34
CD36	0.42 ± 0.09	0.49 ± 0,07a	1.00 ± 0.06	0.78 ± 0.13	0.93 ± 0.05	1.00 ± 0.15	0.79 ± 0.08	0.53 ± 0,06a	1.00 ± 0.05	0.62 ± 0.08	0.57 ± 0.07	1.00 ± 0.06
COX2	1.21 ± 0.23	3.03 ± 0.54	1.00 ± 0.19	0.74 ± 0.18	1.51 ± 0.37	1.00 ± 0.65	1.29 ± 0.20	2.38 ± 0.71	1.00 ± 0.32	1.23 ± 0.27	1.39 ± 0.55	1.00 ± 0.19
CD200	2.41 ± 0,33ab	1.23 ± 0,19b	1.00 ± 0.13	2.05 ± 0,30a	1.41 ± 0.12	1.00 ± 0.20	1.22 ± 0.16	1.10 ± 0.15	1.00 ± 0.05	1.41 ± 0.15	1.20 ± 0.11	1.00 ± 0.13

Relative innate immune gene expression in abdominal SAT, VAT as compared to neck SAT (set to 1) in lean and obese controls and clones, as analyzed by qPCR ± SEM. a= significant difference and fold change of at least ± 1.5 between abdominal SAT/VAT and neck SAT, b = significant difference and fold change of at least ±1.5 between abdominal SAT and VAT.

Effect of obesity

Considering the effect of obesity on liver gene expression in controls and clones, respectively, a mixed effect was seen in the control group (Figure 3A) with 3 out of 8 significantly differentially expressed genes being up-regulated, namely two chemokine genes (IL8 and CXCL10) as well as SFTPA1. The effect of obesity on gene expression in the liver of the cloned pigs (Figure 3B) was limited to only two genes being up-regulated (IL8 and IL18) and HP being down-regulated with all changes being very small. A more limited effect of obesity in cloned as compared to control pigs was also seen on abdominal SAT gene expression as only one gene was down-regulated to a minor degree in obese cloned pigs (TLR4, Figure 3D) while in the obese control group (Figure 3C) five genes were changed compared to lean controls, two of which were up-regulated to a minor degree (APOA1 and CD36). In the neck SAT (Figure 3G) five genes were affected by obesity in control pigs, while only two genes changed expression in cloned pigs (Figure 3H) (down-regulation of ITIH4 and NFKBIA). In VAT, in contrast to the other tissues, a higher number of genes was affected by obesity in the cloned group compared to the control group (Figure 3E and F); in the latter C3 was the only gene affected (minor down-regulation) while four down-regulated genes were observed in the cloned group (IL8, ITIH4, CCL5 and COX-2). As a general trend, all adipose tissue genes changing expression as an effect of obesity in the cloned group were down-regulated while obesity had a mixed effect on adipose tissue gene expression in the control group (Figure 3). The effect of obesity on tissue specific expression patterns, using neck SAT expression as the reference (set to 1) can also be seen in Table 1. The five genes being higher expressed in abdominal SAT of obese controls included one cytokine (IL18) and a group of APPs (C3, CRP, HP and SAA) and the six differentially expressed genes in VAT represented all groups of immune factors.

Figure 3: Control vs. clones – significant genes



*Significantly differentially expressed genes in lean (set to 1) compared to obese in clones and controls with a fold change of ±1.5 in liver (A+B), abdominal SAT (C+D), VAT (E+F) and neck SAT (G+H) as measured with qPCR. For all tissues; controls n=9 and clones n=8 (n=7 in neck SAT). Error bars depict SEM. *=p<0.05, **=p<0.01 and ***=p<0.001.*

In both abdominal SAT and VAT of lean controls, a wide range of innate immune factors, the cytokines IL10, IL18 and TGFB, the APPs C3 and SAA, the chemokine IL8 and the immune related factors CD40, CD200 and TLR4 were differentially expressed with additional differential expression by IL18, CCL2, CCL3L1 and CCL5 in VAT. In obese clones, abdominal SAT showed differential expression of only SAA and IL18 while only IL10 was differentially expressed in VAT. In comparison, lean clones showed differential expression of IL18 and CD200 in both abdominal SAT and VAT with the addition of a group of chemokines (IL8, CCL3L1 and CCL5) in VAT. Finally, genes being differentially expressed between abdominal SAT and VAT were six and eight for lean and obese controls, respectively and two and 0 for lean and obese clones, respectively. Thus, generally, differential tissue expression was reduced by cloning and further reduced by obesity.

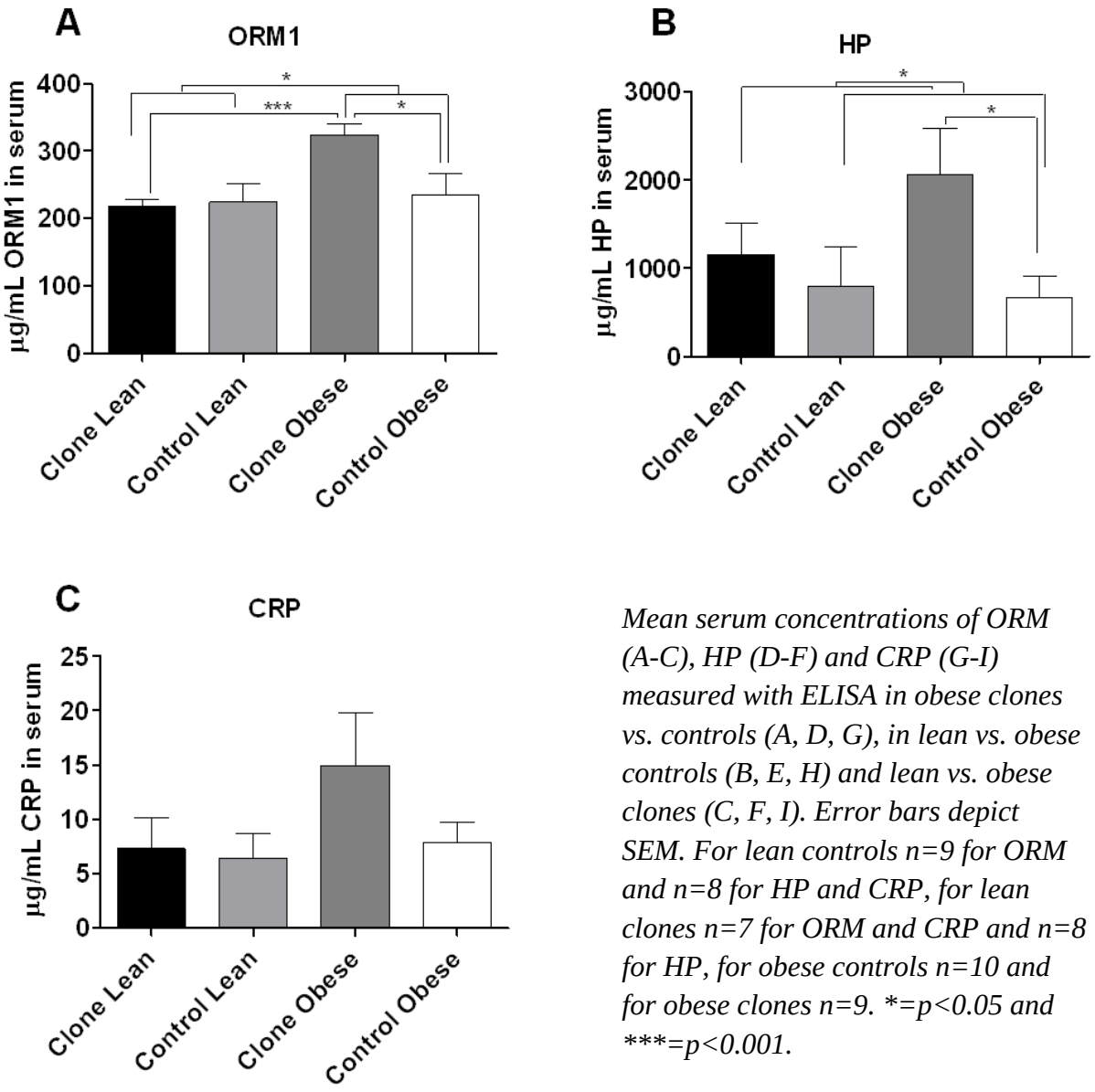
Protein concentrations in serum

Serum protein concentrations of ORM, HP and CRP in the lean and obese controls and clones are shown in Figure 4. IL-6, SAA and TNF-α were below the lower limit of detection for most samples. Possible outlier data points were pin-pointed by the Grubbs test and removed (1 for ORM, 1 for HP and 2 for CRP).

The inter-individual variation in specific serum protein concentrations between animals of the cloned groups was compared to the variation between the animals in the control groups, using the F-test. The variance was found to be not equal for ORM ($p=0.05$) and CRP ($p=0.02$), and bordering on significance for HP ($p=0.053$). However, only for ORM the inter-individual variance was reduced in the clones; for the two other proteins the clones showed higher inter-individual variation than the controls.

The APPs ORM (p=0.03) and HP (p=0.02) had a significantly higher serum concentration in the obese clones compared to the obese controls, being increased approximately 50% and 120%, respectively. CRP also was slightly (although not significantly) elevated in the obese clones. Furthermore, there was a clear and significant higher serum concentration of ORM in obese clones compared to lean clones (p=0.0002), and a tendency for the same to be true for HP and CRP. This was not seen for lean controls compared to obese controls.

Figure 4: Protein quantification in the serum



*Mean serum concentrations of ORM (A-C), HP (D-F) and CRP (G-I) measured with ELISA in obese clones vs. controls (A, D, G), in lean vs. obese controls (B, E, H) and lean vs. obese clones (C, F, I). Error bars depict SEM. For lean controls n=9 for ORM and n=8 for HP and CRP, for lean clones n=7 for ORM and CRP and n=8 for HP, for obese controls n=10 and for obese clones n=9. *=p<0.05 and ***=p<0.001.*

Discussion

Both obese pig groups (controls and clones) in this study had a significantly increased body weight and percentage of body fat compared to both lean groups, however, while the weights of lean controls and lean clones were not significantly different (Rødgaard et al., 2012), cloned obese pigs weighed significantly less than the obese controls and had significantly lower percentage of body fat. Biochemical values reported elsewhere (Christensen et al., 2012) indicate that the feed intake of the obese clones was significantly lower and the daily weight gain correspondingly smaller than seen with the obese controls. As discussed below this potentially could hamper the comparison of adipose gene expression in obese clones with that of obese controls.

It was not possible in this study to demonstrate a lower intra-individual variation in the cloned pigs compared to that of the control pigs for either gene expression or serum protein concentrations, except for the serum concentrations of ORM. This supports other recent findings in pigs cloned by SCNT (Christensen et al., 2012;Clausen et al., 2011;Hwang et al., 2009;Park et al., 2011;Rødgaard et al., 2012;Whyte et al., 2011). For the serum proteins CRP and HP, the trend was that the inter-individual variation was even higher in the cloned group than in the control group.

First, the effect of cloning on innate immune gene expression was studied in the two obese pig groups (clones vs. controls). In the liver (Supplementary Table 1) gene expression was unchanged by cloning except for two significantly differentially up-regulated genes (LBP and SAA), however, the approximately 3 fold up-regulation in liver SAA expression in obese clones was not reflected in serum SAA concentration (data not shown). The same pattern was observed in lean clones compared to lean controls, as SAA expression in the liver was up regulated by 2.5 fold with no concomitant rise in serum SAA concentrations (Rødgaard et al., 2012). In each of the adipose tissues one gene showed a changed expression in obese cloned pigs compared to obese controls (Supplementary Table 1). Overall, the number of genes which was differentially expressed in obese

clones compared to obese controls were smaller than observed in lean clones compared to lean controls, where 2-5 genes were differentially expressed in the adipose tissues (Rødgaard et al., 2012). For tissue expression patterns only very few genes were expressed at significantly different levels in VAT and abdominal SAT compared to neck SAT in the cloned pigs. As discussed below, cloning also reduced differentiated expression in lean pigs (Rødgaard et al., 2012), however not to the same degree as seen in obese pigs.

Second, the response to obesity was compared between clones and controls. In all other tissues than VAT, obesity resulted in fewer gene expression changes in cloned pigs than in control pigs. In cloned pigs all adipose tissue genes affected by obesity were down-regulated, while in the adipose tissue of the control pigs a mixed gene expression response to obesity was seen. The general down-regulation of the adipose tissue response to obesity in the clones observed here is surprising as it does not confirm the elevated levels of immune factors reported to be secreted by adipose tissue in obese subjects (reviewed by for example (Fain, 2010;Weisberg et al., 2003)). Several of the innate immune factors investigated here are known to be secreted by active immune cells in adipose tissue (Fain et al., 2004;Weisberg et al., 2003). Increased infiltration and activity of immune cells are expected in obesity as part of the low-grade adipose-located inflammation implicated in the obesity-related disease syndrome (Bastard et al., 2006;Hotamisligil, 2006), however, one possible reason why the adipose tissue of the obese clones express less chemokines and/or the immune cells might not be activated to the same degree as seen in the controls, could be that obese clones were significantly less obese than the obese controls in this study.

Finally, looking at relative gene expression levels (differential expression) between the three adipose tissue types, neck SAT generally showed the lowest gene expression levels of the immune factors studied here in all four groups of pigs. More genes were more highly expressed in either VAT or abdominal SAT or both in the controls compared to the clones. In the obese clones this

could be explained by the difference in percentage of body fat between the obese clones and controls (Figure 1 and 2), however, this does not explain the lowered gene expression changes in lean clones compared to lean controls. The weights of lean clones and controls were similar, even though a significantly different daily weight gain was observed, and the two groups shows a very similar response to the diet, as reported by (Christensen et al., 2012). Therefore, it would seem that the cloning procedure in itself is a factor in limiting the differentiation of innate immune response genes.

Differences between controls and cloned animals were also observed for the serum concentrations of APPs, including ORM and HP (with CRP showing the same tendency), as obese, cloned pigs showed significantly increased serum concentrations of those APPs. It is of interest to note that the liver expression of HP was in fact down-regulated in obese clones compared to lean clones and not found to be changed in any other tissue; thus the observed increase in serum HP concentration must be ascribed to other, unknown sources. Blood plasma concentration of CRP, SAA and IL-6 were found to be elevated in overweight women with cardiovascular disease (Ridker et al., 2000) and serum concentrations of ORM and HP were increased in obese humans (Chiellini et al., 2004;Lee et al., 2010) so taken alone the cloned pigs seem to reflect this obesity response better than the control pigs, even if pro-inflammatory cytokines like TNF-α and IL-6, classically involved in the low-grade inflammation of obesity (reviewed in (Tataranni and Ortega, 2005)) were not detected in either obese controls or obese clones (data not shown).

Overall, cloning decreased the number of genes responding to obesity in all tissues (except VAT) investigated and also decreased the number of genes being differentially expressed in the three different adipose tissues investigated, both in lean and obese pigs. There was a small difference in the genetic background of the clones (65% Landrace; 35% Yorkshire) and the two control crossbreeds (36%:64% and 75%:25%) however it may be assumed that this small genetic difference

414 will not by itself lead to the observed gene expression differences between clones and controls. This
415 is supported by the fact that the two genetically slightly different control cross breeds did not show
416 any difference in reference gene expression.
417 One possible explanation for the more limited gene expression changes seen in obese cloned
418 animals as opposed to obese control animals is the lower feed intake of the cloned pigs (Christensen
419 et al., 2012) and the consequently lower final weight than the obese controls, simply leading to a
420 reduced “obesity effect” on the animal. Interestingly, however, we did find changes (increases) in
421 the serum concentrations of APPs in the obese, cloned pig group compared to the other three groups
422 while control pigs showed obesity induced changes in the expression in a number of innate immune
423 system related genes in all of the four tissues investigated. These controls, however, did not show
424 any change in circulating APP or cytokine serum concentrations as a response to obesity. Taken
425 together this may indicate that the less differentiated and more constant expression of innate
426 immune genes in the cloned pigs might result in a decreased ability to cope with obesity as
427 indicated by the observable changes in APP concentrations. This is probably related to
428 inflammation and is originating from an extra-hepatic source.
429 In summary, as in the lean pigs (Rødgaard et al., 2012) cloning did not reduce inter-individual
430 variability in innate immune gene expression between obese pigs. Furthermore, cloning influenced
431 the expression of 1-2 innate immune genes in each of the tissues of obese clones compared to obese
432 controls. In light of these results and other published data (Christensen et al., 2012;Clausen et al.,
433 2011;Jensen et al., 2010) it can be concluded that cloning does not increase the usability of pigs as
434 model animals for innate responses to diet-induced obesity, even though cloned pigs showed an
435 increase in serum APP concentrations in agreement with human studies.
436

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Author disclosure statement

The authors declare that no conflicting financial interests exist.

4 Study II – Göttingen and Ossabaw minipigs

As explained in Chapter 3 the obese phenotypes pigs from the Nutriomics project were not utilized in the work for this thesis and we therefore had to locate other pigs that could serve as such. As a result, we have established collaborations with groups and universities that have made two obese phenotype pigs available to us; namely the Göttingen minipig and the Ossabaw minipig.

4.1 Background

Samples from the Göttingen minipigs were made available through a collaboration with Novo Nordisk A/S and the University of Copenhagen. Seven female obese Göttingen minipigs had been used in therapeutic studies investigating the effect of different therapeutic peptides on obesity by Novo Nordisk. As the pigs were nearing termination, Novo Nordisk A/S offered them to be investigated by different groups and universities. Seven female lean age matched controls were purchased from Ellegaard Göttingen Minipigs A/S (Dalmose, Denmark) where the obese pigs had been purchased originally as well. All pigs were ovariectomized. Obesity was induced by *ad libitum* feeding and the lean pigs received 2 x 150 g gram a day. Both groups were fed standard minipig chow, so the obese pigs were not fed high fat, high sugar or high cholesterol diets. Nonetheless, the obese minipigs weighed significantly more and had a significantly higher body fat percent than the lean pigs, as evident by the pictures in Figure 10. For phenotypic characterization of these pigs, see Table 10, Paper III p. 70 and Paper IV p. 95. The pigs were housed at the stables at the University of Copenhagen (Taastrup, Denmark). Several investigations have been performed in these pigs so far, however, none have been published yet.

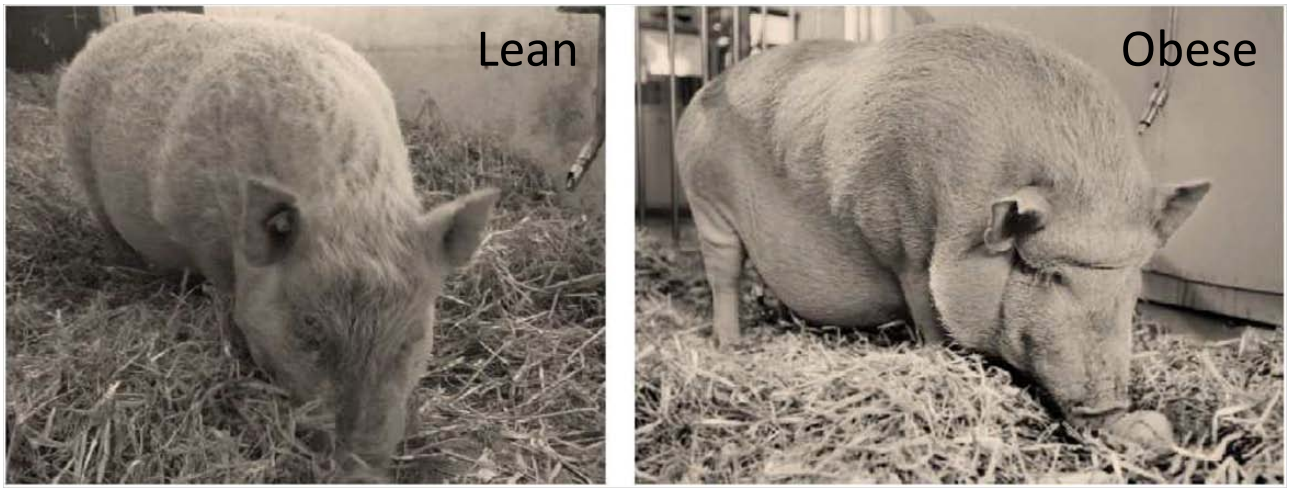

Figure 10: Pictures of a lean and an obese Göttingen minipig. Courtesy of the University of Copenhagen and Novo Nordisk A/S.

Samples from the Ossabaw minipigs were made available through a collaboration with Professor Michael Sturek and his group at the University of Indiana. Samples from several studies were made available to us, however, as we were interested in the effects of obesity, only samples from one study into obesity and polycystic ovary syndrome (PCO) was used (the Ossabaw14 study). The study comprised of 12 female pigs; seven lean and five obese, however, we were not supplied with samples from all pigs: We received a full set of samples from abdominal SAT and VAT, no samples from the neck SAT, liver samples from only six lean pigs, and serum samples from six lean and four obese pigs. The lean pigs were fed a standard chow (5L80)

and the obese pigs were fed an atherogenic diet. Both diets are further described in (Lee *et al.* 2009). All pigs were obtained from the Indiana University-Purdue University Indianapolis Medical Center and they were reared and housed at Purdue University breeding colony (West Lafayette, IN, USA). Obese Ossabaw minipigs weighed significantly more than the lean pigs, as depicted in Figure 11. For phenotypic characterization of these pigs, see Paper IV p. 95, Table 10 and (Lee *et al.* 2009).

Figure 11: Pictures of a lean and an obese Ossabaw minipig. Source: (Kreutz *et al.* 2011)

4.1.1 Göttingen and Ossabaw minipigs as model animals for nutritional studies

The Ossabaw and Göttingen breeds of minipigs have been thoroughly investigated as models for obesity-induced metabolic pathologies.

4.1.1.1 The Göttingen minipig

The Göttingen minipig is developed by crossbreeding the Vietnamese swine, the Hormel, and German improved Landrace swine in the 1960s at the Institute of Animal Breeding and Genetics at the University of Göttingen in Germany. It has been developed and used extensively as a model for diabetes by researchers at Novo Nordisk A/S, among others, and the use of Göttingen as a model for diabetes is reviewed in (Larsen & Rolin 2004). Both female and male Göttingen minipigs become obese when fed a high-fat high-energy diet, with increased body weights and body fat (Bollen *et al.* 2005). When compared to males, female Göttingen minipigs had significantly higher body weights, were more obese and had a more atherogenic plasma profile than male minipigs, therefore, female Göttingen minipigs have been suggested to be better models for MetS than male minipigs (Bollen *et al.* 2005; Christoffersen *et al.* 2007). Furthermore, age have an impact on plasma concentrations of several metabolic factors, including increased levels of triglycerides and glucose, in both female and male pigs (Christoffersen *et al.* 2007; Larsen *et al.* 2001).

4.1.1.2 The Ossabaw minipig

The Ossabaw minipig is a feral breed found on the Ossabaw Island, GA, USA. It is believed that the population on the island is derived from pigs being left on the island in the 1500s by Spanish explorers, and since then have been subjected to a selection pressure of seasonal feast and famine ecology of the Ossabaw Island, which has led to the adaptation of a "thrifty genotype" in the pigs (Sturek *et al.* 2007). The thrifty genotype hypothesis is that humans have had to develop an ability to store excess fat in the hunter-gatherer stages of human development in order to survive periods of famine (Neel 1962). Due to this, and

other unique adaptations to their natural habitat, the Ossabaw minipigs were studied extensively in the 1970s and 1980s, as described in (Sturek *et al.* 2007). In the beginning of the 2000s, the Ossabaw minipig sparked renewed interest because of the obesity and diabetes epidemic, and a breeding colony was established at the University of Indiana, USA, after an expedition to the island in 2002.

When compared to Yorkshire pigs which is a lean phenotype pig, it was found that Ossabaws have more back fat; 2.64 cm in Yorkshire, and 5.97 cm in age-matched Ossabaws (Hausman & Martin 1981), and when dietary restricted to 65% of *ad libitum* intake, the Yorkshire had a 7-fold decrease in adipose tissue, where Ossabaw pigs only had a 2.6-fold decrease in adipose tissue (Etherton *et al.* 1982; Etherton & Kris-Etherton 1980). When fed excess calories, the Ossabaw minipigs develops several symptoms of the MetS, including intra-abdominal obesity, insulin resistance and impaired glucose tolerance (Wangsness *et al.* 1977; Wangsness *et al.* 1980), as well as increased triglyceride and cholesterol levels in the blood (Etherton & Kris-Etherton 1980), when compared to lean Ossabaw pigs. As these studies were done before the MetS was recognized as a serious health problem, several studies were done to confirm the metabolic characteristics of male and female Ossabaw minipigs in the 2000s (Dyson *et al.* 2006; Neeb *et al.* 2010; Lee *et al.* 2009) and it was found that female obese Ossabaw minipigs had 5 characteristics of the MetS (Dyson *et al.* 2006). Therefore the Ossabaw minipigs has been proposed as one of the best porcine models for obesity, MetS and diabetes not induced by chemicals (Bellinger *et al.* 2006).

4.2 Hypothesis for Study II

- Lean and obese Ossabaw and Göttingen minipigs show differential tissue and blood serum expression of innate immune factors
- In the Göttingen minipigs the adipose tissue from the abdomen; retroperitoneal adipose tissue (RPAT) and VAT, have a higher number of innate immune response genes that are affected by obesity than the neck SAT
- The Ossabaw and Göttingen minipigs have similar responses to obesity in terms of the innate immune response as humans

4.3 Results for Study II – Göttingen minipigs

4.3.1 Pre-PCR data for Göttingen minipigs

Tissue	ng/µl	260/230	260/280	RIN
Liver	129-1418 (413)	1.87-2.20 (2.06)	2.02-2.13 (2.08)	8.3-9.5 (9.0)
RPAT	58-811 (264)	0.28-2.16 (1.45)	1.85-1.97 (1.78)	2.5-8.2 (7.1)
VAT	48-739 (510)	0.94-2.15 (1.53)	1.63-2.06 (1.93)	2.4-6.8 (5.5)
Neck SAT	41-606 (252)	0.50-2.03 (1.53)	1.58-1.97 (1.79)	5.4-7.5 (6.8)

Table 6: Yields, ratios and RINs of the Göttingen minipigs in the four measured tissues. The lowest and highest values are shown, with the mean in parenthesis. n=14 for all tissues.

The yields and quality measurements of the 14 pigs and 4 tissues as measured with the NanoDrop and Bioanalyzer (discussed in section 2.1.1) are shown in Table 6. The yields were high in all tissues and the 260/230 and 260/280 ratios and RINS showed high quality RNA for liver. All adipose tissues showed low 260/230 ratios, and RPAT and neck SAT showed 260/280 ratios averaged just below the accepted 1.8 (as described in section 2.1.1). The RINs for especially VAT were low (5.5), but over 5, which was described in section 2.1.1 as good quality RNA.

4.3.2 Paper III

Characterization of gene expression in obese Göttingen minipigs: Extensive gene expression changes in liver and adipose tissue in otherwise well-adapted, obese Göttingen minipigs

Rødgaard T, Skovgaard K, Moesgaard, SG, Christoffersen BØ, Heegaard PMH. J Anim Sci. 2012, submitted as original paper

The layout of the article is changed compared to the submitted version.

Even though the Göttingen minipig has been investigated extensively as a model for obesity and diabetes (section 4.1.1.1), it has not yet been investigated how the immune system is affected by obesity in these pigs, and how similar the response is to the human response. Therefore, we tested a panel of 35 genes in the liver and 33 genes in the adipose tissue related to the innate immune system with high-throughput chip-based qPCR (Fluidigm), as well as five innate immune serum proteins in the blood with ELISA.

The study revealed extensive gene expression changes in the tissues between lean and obese pigs, as nine genes had significantly differential expression in the liver, 11 in the abdominal SAT, 12 in the VAT and eight in the neck SAT. Of these, three genes were significantly differentially expressed in all three adipose tissues in obese pigs; proinflammatory *CCL3L1* was upregulated, anti-inflammatory *CD200* was downregulated and the proinflammatory antagonist *IL1RN* was upregulated, which corresponds to obese humans. However, the Göttingen minipigs did not show a differentiated serum protein response which does not correspond to human serum protein response to obesity.

Characterization of gene expression in obese Göttingen minipigs: Extensive gene expression changes in liver and adipose tissue in otherwise well-adapted, obese Göttingen minipigs[1]

Tina Rødgaard(*), Kerstin Skovgaard(*), Sophia G. Moesgaard(§(presently #)), Susanna Cirera (§), Berit Ø. Christoffersen(#), Peter M. H. Heegaard(*)[2]

*Innate Immunology Group, National Veterinary Institute, Technical University of Denmark, Frederiksberg C, Denmark §Department of Veterinary Clinical and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg C, Denmark #Novo Nordisk A/S, Maaløv, Denmark

Running head: Innate immune gene expression in obese pigs

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[2] Corresponding author: Professor Peter M. H. Heegaard, Innate Immunology Group, National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, 1870 Frederiksberg C, Denmark. E-mail: pmhh@vet.dtu.dk

ABSTRACT

The usability of Göttingen minipigs as models for obesity and obesity-related pathologies is well-established. The low-grade inflammation associated with obesity involves a range of innate immune factors, however, to our knowledge, the impact of obesity on innate immune factor expression has not been studied in Göttingen minipigs. Therefore, we studied the expression of innate immune genes in liver and adipose tissues, and serum concentrations of cytokines and acute phase proteins in obese vs. lean Göttingen minipigs. In the liver, out of 35 investigated genes, the expression of 9 was significantly different in obese pigs. Of 33 genes in adipose tissues obesity changed the expression of 12 genes in the visceral adipose tissue (VAT), 11 in the abdominal retroperitoneal adipose tissue (RPAT), and 8 in the subcutaneous adipose tissue (SAT) from the neck. Three genes were differentially expressed in all adipose tissues, namely the CC chemokine ligand 3-like 1 (CCL3L1), CD200 and interleukin 1 receptor antagonist (IL1RN) with IL1RN being the most highly regulated gene in both VAT and abdominal SAT. In contrast to obese humans, no changes in serum concentrations of HP, CRP, SAA, TNF-α and IL-6 were found in obese Göttingen minipigs.

KEYWORDS

Göttingen minipigs, obesity, gene expression, innate immune response, mRNA.

INTRODUCTION

Obesity has become an important health problem worldwide and is associated with elevated risks of several life-threatening conditions, including cardiovascular diseases and type II diabetes. Obesity is associated with chronic low-grade activation of the innate immune system, resulting in a chronic

inflammation of the adipose tissue which is thought to lead to the development of these disease states (Bastard et al., 2006; Federico et al., 2010; Gil et al., 2007). A wide range of factors of the innate immune response have been investigated with regards to obesity (reviewed for example in (Gil et al., 2007) and (Federico et al., 2010)). In the search for good animal models of obesity, the pig has been proposed as an excellent biomedical model as it is very similar to man with regards to anatomy, physiology, biochemistry and metabolism (Litten-Brown et al., 2010). The Göttingen minipig has been proposed as a useful model of human obesity (Johansen et al., 2001) and diabetes (Larsen et al., 2007; Larsen et al., 2002). To further investigate the usability of the Göttingen minipig as a human obesity model, it is important to investigate other molecular parameters associated with obesity. The objective of this study was therefore to elucidate the effects of diet-induced obesity on the gene expression of innate immune factors in the liver and 3 types of adipose tissues in the Göttingen minipig and relate this to human obesity.

MATERIALS AND METHODS

The study was approved by the Danish Animal Experiments Inspectorate. Fourteen female, ovariectomized Göttingen minipigs (7 lean and 7 obese) originating from Ellegaard Göttingen Minipigs A/S (Dalmose, Denmark) were included in the study. The pigs were housed at University of Copenhagen (Taastrup, Denmark) from 1-2 yr of age and forward. The obese pigs had previously been used in pharmacological studies with therapeutic peptides, but had been subjected to a suitable wash-out period prior to this study. Lean pigs were fed minipig standard chow; Altromin 9023 2x150 g a day and obese pigs were fed minipig standard chow; Altromin 9033 ad libitum. The minipigs were euthanized at 41-47 mo of age with pentobarbital and desangiunated. Tissues were collected from the liver, subcutaneous adipose tissue (SAT) from the neck, mesenteric adipose

tissue (VAT), and abdominal cranioventral retroperitoneal adipose tissue (RPAT) and snap frozen in liquid nitrogen, and blood was collected for serum preparation. Tissue and serum samples were kept at -80°C until analysis.

Extraction of RNA

All samples were homogenized on a gentleMACS Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) in gentleMACS M tubes (Miltenyi Biotec). For liver samples RNA was extracted using the RNeasy Lipid tissue Midi kit (Qiagen, Copenhagen, Denmark) according to manufacturer's protocol. All samples were subjected to on-column RNase-free DNase digestion (Qiagen).

For the adipose tissues an optimized procedure was developed as follows: RNA was extracted with phenol and chloroform: Approximately ½ g of adipose tissue was homogenized with 4 mL of QIAzol Lysis Reagent (Qiagen) on the gentleMACS Dissociator and then placed at room temperature for 5 min. The homogenate was centrifuged at 12.000xg for 10 min at 4°C after which the fatty upper layer was removed. Chloroform (Merck, NJ, USA) was mixed with the remaining supernatant and placed at room temperature for 3 minutes after vigorous shaking for 15 s. 1 mL of isopropanol (2-propanol, Merck) was added to the upper, aqueous phase and was centrifuged at 12000xg for 10 min at 4°C after 5 min at room temperature. The pellet was washed 2 times in cold 75% ethanol, with a centrifugation step of 8 min at 8000xg at 4°C between each wash and then dried at 55°C for 2 min. The RNA was dissolved in RNase-free water at 55°C for 10 min.

For all samples the RNA yield was measured on the NanoDrop (ND-1000 Spectrophotometer, NanoDrop Technologies Inc, USA) and RNA integrity was measured with the Agilent RNA 6000 Nano Kit (Agilent Technologies, CA, USA) on the Agilent 2100 Bioanalyzer (Agilent Technologies) according to the manufacturer's protocol. Liver samples yielded RNA with an RNA

integrity numbers (RINs) of 9.0±0.1, RPAT samples had RINs of 7.1±0.4, VAT sample RINs was 5.5±0.4, and neck SAT samples had RINs of 6.8±0.2.

Gene Expression

The gene expression was performed as described in Rødgaard et al. Briefly, cDNA syntheses of the samples was performed in duplicate (technical replicates) using 500 ng of total RNA. This was reverse transcribed using the QuantiTECT Reverse Transcription kit (Qiagen), according to the manufacturer's instructions. Pre amplification was performed using TaqMan PreAmp Master Mix (Applied Biosystems, CA, USA).

Primers were designed as described previously in (Skovgaard et al., 2009). Gene symbols, primer sequences and amplicon lengths are shown in Table 1. Primer amplification efficiencies, dynamic ranges, specificity and sequences were analyzed as described in (Rødgaard et al., 2012).

Quantitative real-time PCR was performed in 48.48 Dynamic Array Integrated Fluidic Circuits (Fluidigm, CA, USA), combining 48 samples with 48 primer sets in the BioMark real-time PCR instrument (Fluidigm). Expression data were acquired using the Fluidigm Real-Time PCR Analysis software 3.0.2 (Fluidigm) and exported to GenEx5 ((MultiD, Göteborg, Sweden).

Table 1: Primers used for high-throughput qPCR

Gene symbol	Gene name	Sequence	Amplicon length
AOAH	Acyloxyacyl hydrolase	F: GTAATGGCATTTGGGGTGTC R: TCTCCCAGCAAAATGATTCC	97
ADIPOQ	Adiponectin	F: AACATGCCCATTCGCTTTAC R:AGACCGTGATGTGGAAGGAG	121
ORM1(a)	Orosomucoid 1	F: AGTCCTGAGCCTCCTTCCTC R: GCCGAGCCGATATAATACCA	123
ORM1(b)	Orosomucoid 1	F: ACCCCCAGTACAATGAGTCG R: TTAACAGCAGGTCAGCAACG	210
APOA1	Apolipoprotein A-I	F: GTTCTGGGACAACCTGGAAA R: GCTGCACCTTCTTCTTCACC	86
B2M	Beta-2-microglobulin	F: TGAAGCACGTGACTCTCGAT R: CTCTGTGATGCCGGTTAGTG	70
ACTB	Beta-actin	F: CTACGTCGCCCTGGACTTC	76

			R: GCAGCTCGTAGCTCTTCTCC	
CRP	C-reactive protein	F: GGTGGGAGACATTGGAGATG		85
			R: GAAGGTCCCACCAGCATAGA	
CD14	CD14	F: GGGTTCCTGCTCAGATTCTG		164
			R: CCCACGACACATTACGGAGT	
CD36	CD36	F: GCCTATCCTCTGGCTTAATGAG		135
			R: AACATCCCCACACCAACACT	
CD40	CD40	F: TGAGAGCCCTGGTGGTTATC		90
			R: GCTCCTTGGTCACCTTTCTG	
CD163	CD163	F: CACATGTGCCAACAAAATAAGAC		130
			R: CACCACCTGAGCATCTTCAA	
CD200	CD200	F: TCCCCAGGAAGTTTTGATTG		84
			R: CCATGGTTCTTGCTGAAGGT	
CCL2	Chemokine (C-C motif) ligand 2	F: GCAAGTGTCCTAAAGAAGCAGTG		103
			R: TCCAGGTGGCTTATGGAGTC	
CCL3L1	Chemokine (C-C motif) ligand 3-like 1	F: CCAGGTCTTCTCTGCACCAC		90
			R: GCTACGAATTTGCGAGGAAG	
CCL5	Chemokine (C-C motif) ligand 5	F: CTCCATGGCAGCAGTCGT		121
			R: AAGGCTTCCTCCATCCTAGC	
CXCL10	Chemokine C-X-C motif ligand 10	F: CCCACATGTTGAGATCATTGC		141
			R: GCTTCTCTCTGTGTTCGAGGA	
C3	Complement component 3	F: ATCAAATCAGGCTCCGATGA		76
			R: GGGCTTCTCTGCATTTGATG	
PBD2	Defensin, beta 2	F: CAGGATTGAAGGGACCTGTT		99
			R: CTTCACTTGGCCTGTGTGTC	
FIB	Fibrinogen	F: GAATTTTGGCTGGGAAATGA		86
			R: CAGTCCTCCAGCTGCACTCT	
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F: ACCCAGAAGACTGTGGATGG		79
			R: AAGCAGGGATGATGTTCTGG	
HP	Haptoglobin	F: ACAGATGCCACAGATGACAGC		105
			R: CGTGCGCAGTTTGTAGTAGG	
HPRT1	Hypoxanthine phosphoribisyl transferase I	F: ACACTGGCAAAACAATGCAA		71
			R: TGCAACCTTGACCATCTTTG	
IL1B	Interleukin 1, beta	F: CCAAAGAGGGACATGGAGAA		123
			R: GGGCTTTTGTTCTGCTTGAG	
IL6(a)	Interleukin 6	F: TGGGTTCAATCAGGAGACCT		116
			R: CAGCCTCGACATTTCCCTTA	
IL6(b)	Interleukin 6	F:CCTCTCCGGACAAAACTGAA		118
			R:TCTGCCAGTACCTCCTTGCT	
IL8(a)	Interleukin 8	F: GAAGAGAACTGAGAAGCAACAACA		99
			R: TTGTGTTGGCATCTTTACTGAGA	
IL8(b)	Interleukin 8	F: TTGCCAGAGAAATCACAGGA		78
			R: TGCATGGGACACTGGAAATA	
IL10(a)	Interleukin 10	F: CTGCCTCCCACTTTCTCTTG		95
			R: TCAAAGGGGCTCCCTAGTTT	
IL10(b)	Interleukin 10	F: TACAACAGGGGCTTGCTCTT		110
			R: GCCAGGAAGATCAGGCAATA	
IL12A	Interleukin 12A	F: CCACCTGGACCATCTCAGTT		94
			R: CAGCAGATTTTGGGAGTGGT	
IL18	Interleukin 18	F: CTGCTGAACCGGAAGACAAT		100
			R: TCCGATTCCAGGTCTTCATC	
IL1RN	Interleukin 1 receptor antagonist	F: TGCCTGTCCTGTGTCAAGTC		90
			R: GTCCTGCTCGCTGTTCTTTC	
ITIH4(b)	Inter-alpha-trypsin inhibitor heavy chain family, member 4	F: AGGCCCTCACCATATCACAG		110
			R: GTTGCCATCCAGGACTGTTT	
ITIH4(a)	Inter-alpha-trypsin inhibitor heavy	F: ATGACAGCAAGCGAACAGTG		85

	chain family, member 4	R: GGGGATCCCTCTTGGTAATC	
IFNG	Interferon gamma	F: CCATTCAAAGGAGCATGGAT	76
		R: TTCAGTTTCCCAGAGCTACCA	
LBP	Lipopolysaccharide binding	F: CCCAAGGTCAATGATAAGTTGG	83
	protein	R: ATCTGGAGAACAGGGTCGTG	
NFKB1	Nuclear factor of kappa light	F: CTCGCACAAGGAGACATGAA	97
	polypeptide enhancer in B-cells 1	R: GGGTAGCCCAGTTTTTGTCA	
NFKBIA	Nuclear factor of kappa light polypeptide	F: GAGGATGAGCTGCCCTATGAC	85
	gene enhancer in B-cells inhibitor, alpha	R: CCATGGTCTTTTAGACACTTTCC	
PAFAH1B1	Platelet-activating factor	F: GCAAACTGGCTACTGTGTGAAG	113
	acetylhydrolase 1b	R: GCACAGTCTGGTCATTGGAA	
COX2	Prostaglandin-endoperoxide	F: AGGCTGATACTGATAGGAGAAACG	100
	synthase 2	R: GCAGCTCTGGGTCAAACTTC	
RPL13A	Ribosomal protein L13a	F: ATTGTGGCCAAGCAGGTACT	76
		R: AATTGCCAGAAATGTTGATGC	
SAA	Serum amyloid A	F: TGGAGAGCCTACTCGGACAT	90
		R: CCTTTGGGCAGCATCATAGT	
SFTPA1	Surfactant protein A1	F: CATGGGTGTCCTCAGTTTCC	86
		R: CATCAAAAGCGACTGACTGC	
TLR4	Toll-like receptor 4	F: TTTCCACAAAAGTCGGAAGG	145
		R: CAACTTCTGCAGGACGATGA	
TF	Transferrin	F: CTCAACCTCAAAACTCCTGGAA	82
		R: CCGTCTCCATCAGGTGGTA	
TGFB1	Transforming growth factor beta 1	F: GCAAGGTCCTGGCTCTGTA	97
		R: TAGTACACGATGGGCAGTGG	
TNF	Tumor necrosis factor	F: CCCCCAGAAGGAAGAGTTTC	92
		R: CGGGCTTATCTGAGGTTTGA	
TNFAIP3	Tumor necrosis factor	F: CCCAGCTTTCTCTCATGGAC	113
	alpha-induced protein 3	R: TTGGTTCTTCTGCCGTCTCT	

a and b denotes different primer pair annealing to the same mRNA transcript

Serum Protein Quantification

Serum protein concentrations were determined using sandwich ELISA. Commercially available ELISA assays were used for IL-6 (Porcine IL-6 Duoset kit, R&D Systems, inc., MN,USA), TNF-α (Swine TNF-α CytoSetTM, Invitrogen, CA, USA, with Antibody Pair Buffer Kit (Invitrogen)) and SAA (Phase SAA assay, Tridelta Development Ltd., Kildare, Ireland) according to manufacturer's protocol. CRP and HP were analyzed with in-house assays as described in (Rødgaard et al., 2012). TMB-plus (KEM-EN-TEC, Taastrup, Denmark) was used for developing the ELISA-plates, according to the manufacturer's instructions, which were then read using an automatic plate reader (Thermo Multiskan Ex spectrophotometer, Thermo Scientific, MA, USA). Ascent software v. 2.6, Thermo Scientific was used to calculate sample values calibrating each plate with several dilutions

of a standard solution of known concentration of the protein in question. Outliers were identified with Grubbs outlier test (Grubbs, 1969) and removed from the dataset where applicable.

Data Analysis and Statistics

For qPCR data pre-processing, normalisation and relative quantification GenEx5 was used. Out of a panel of 5 putative reference genes the most stably expressed were selected for normalisation (for liver: GAPDH, B2M, RPL13A, and ACTB, and for the adipose tissues all 5 were chosen: RPL13A, HPRT1, GAPDH, B2M, and ACTB) using GeNorm (Vandesompele et al., 2002). The geometric mean of these was used for normalization of each tissue individually in GenEx5. To calculate relative expression levels, the Cq values were calculated relative to the least expressed sample for each primer set and log2 transformed to approach normal distribution prior to two-tailed, unpaired t-test, one-way ANOVA with Tukey-Kramer post test, and/or F-test. Statistics were performed in GenEx5 or Prism5 (GraphPad Software, Inc., CA, USA). For ELISAs significance was tested using a two-tailed unpaired t-test and variance was tested with an F-test. $p \leq 0.05$ was considered significant. Data are expressed as the mean ± SEM.

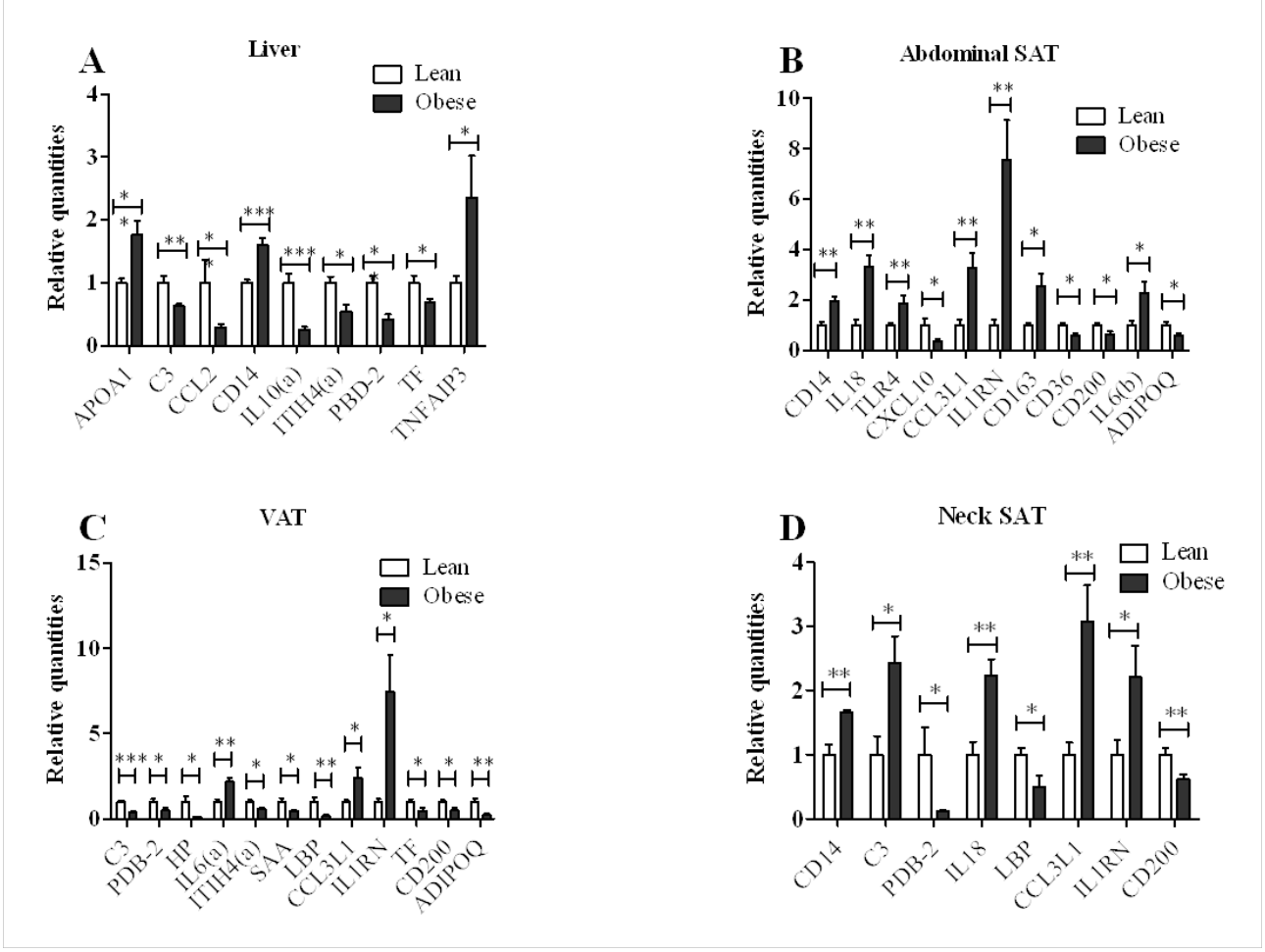
RESULTS

Gene Expression

The expression profiles of 39 genes related to innate immunity were analyzed as well as 5 putative reference genes. In the pre processing of data, several genes were removed from further analysis due to low efficiencies or due to high variation between the technical replicates (as described in (Rødgaard et al., 2012)): FIB, IL1B, IL8(a), IL8(b), AOAH, IL10(b), ORM1(a), ORM1(b),

SFTPA1 and IFNG were removed from the fatty tissue data and IL1B, AOAH, SFTPA1, ORM1(a), ORM1(b), ADIPOQ, LBP and IL8(a) from the liver tissue data.

Figure 1: Significant differentially expressed genes in lean compared to obese Göttingen minipigs



*Significantly expressed genes in lean (set to 1) compared to obese Göttingen minipigs with a fold change equal to or greater than ±1.5 in liver (A), RPAT (B), VAT (C) and neck SAT (D) as determined with qPCR. In the VAT, both primers for IL6 showed similar patterns and significance (**), therefore IL6(b) was removed from the graph. For all tissues; lean n=7 and obese n=7. Error bars depict SEM. *=p<0.05, **=p<0.01 and ***=p<0.001.*

Genes differentially expressed between lean and obese pigs at significance level p=0.05 and with a change of at least ± 1.5 fold are depicted for all four tissues in Fig. 1. Overall, obesity resulted in a significantly changed expression of 12 genes in VAT (9 of which were down-regulated in obese

247 animals), 11 genes in RPAT (of which 7 were up-regulated in the obese group), and 8 genes in neck
248 SAT (3 of these genes being down-regulated). The genes significantly affected by obesity in the
249 liver were mostly down-regulated (6 out of 9).

250 Three genes were significantly differentially regulated between the lean and obese minipigs in all 3
251 adipose tissues, namely CCL3L1, IL1RN and CD200, with CCL3L1 and IL1RN being up-regulated
252 and CD200 being down-regulated in all three adipose tissue types of obese pigs. In the liver, none
253 of these three genes were affected by obesity.

254 The pattern of tissue specific adipose tissue gene expression may also be compared between lean
255 and obese pig, see Table 2.

256 For both obese and lean pigs approximately half of the investigated genes showed different
257 expression levels in the three types of adipose tissues. In the lean group, all of these genes were
258 expressed at higher levels in RPAT and VAT than in neck SAT (19 genes), while in the obese pigs
259 the proportion of genes being up-regulated in RPAT and VAT compared to neck SAT was lower
260 (10 genes). Specifically, in the obese group, more APPs were significantly differentially regulated
261 between adipose tissues, with 6 genes (C3, CRP, HP, ITIH4(a), ITIH4(b), LBP and SAA) compared
262 to two genes (C3 and CRP) in the lean minipigs; in contrast only 2 cytokines (TGFB1 and IL1RN)
263 and no chemokines were significantly differentially regulated between adipose tissues in the obese
264 animals, compared to 4 cytokines (IL10, IL18, TGFB1 and TNFAIP3) and 2 chemokines (CCL3L1
265 and CXCL10) in the adipose tissues of lean minipigs.

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Table 2: Innate immune gene expression in adipose tissues

	Lean			P-values:			Obese			P-values:		
Genes	RPAT	VAT	Neck SAT	a	b	c	RPAT	VAT	Neck SAT	a	b	c
ADIPOQ[1]	36.75 ± 4.36	19.39 ± 3.97	16.32 ± 2.15	0.11	0.05	1.00	22.35 ± 2.61	4.65 ± 1.31	12.97 ± 1.19	<0.001	0.39	<0.001
APOA1[2]	2.08 ± 0.32	4.41 ± 0.84	3.91 ± 0.63	0.97	1.00	1.00	7.17 ± 2.43	5.26 ± 0.50	5.93 ± 0.93	0.97	0.91	1.00
C3[2]	10.54 ± 5.14	249.09 ± 20.89	3.71 ± 1.09	<0.001	0.15	<0.001	46.95 ± 15.21	93.50 ± 12.69	9.02 ± 1.55	0.27	0.12	<0.001
CCL2[3]	4.19 ± 0.85	5.71 ± 0.93	3.06 ± 0.60	0.87	0.89	0.27	4.35 ± 0.67	7.27 ± 1.89	5.61 ± 1.30	0.98	1.00	0.97
CCL3L1[3]	5.52 ± 1.28	11.36 ± 1.15	6.62 ± 1.23	0.05	0.95	0.28	18.13 ± 3.20	27.66 ± 6.40	20.37 ± 3.73	0.82	1.00	0.97
CCL5[3]	3.67 ± 0.67	5.86 ± 0.42	3.61 ± 0.69	0.19	1.00	0.22	6.81 ± 2.07	4.50 ± 0.34	5.12 ± 0.60	1.00	1.00	1.00
CD14[4]	2.22 ± 0.31	4.52 ± 0.71	1.85 ± 0.29	0.005	0.87	<0.001	4.31 ± 0.39	3.99 ± 0.36	3.06 ± 0.09	1.00	0.74	0.93
CD163[4]	4.37 ± 0.39	10.02 ± 2.04	1.90 ± 0.34	0.28	0.008	<0.001	11.01 ± 2.30	13.52 ± 1.85	3.53 ± 0.91	0.90	0.001	<0.001
CD200[4]	6.33 ± 0.54	6.91 ± 0.85	2.52 ± 0.25	1.00	0.03	0.02	4.02 ± 0.70	3.53 ± 0.73	1.55 ± 0.19	0.96	0.006	0.051
CD36[4]	5.73 ± 0.56	2.16 ± 0.38	2.64 ± 0.23	<0.001	<0.001	0.74	3.45 ± 0.40	1.78 ± 0.16	2.47 ± 0.21	0.03	0.26	0.88
CD40[4]	2.35 ± 0.27	4.68 ± 0.21	1.44 ± 0.15	0.002	0.03	<0.001	2.97 ± 0.32	5.44 ± 0.49	2.71 ± 0.31	0.00	0.99	<0.001
COX-2[4]	4.54 ± 0.57	6.65 ± 0.52	2.97 ± 0.47	0.63	0.50	0.02	3.87 ± 0.66	5.72 ± 0.99	4.88 ± 0.89	0.97	0.96	1.00
CRP[2]	11.38 ± 2.64	7.11 ± 1.26	3.44 ± 0.59	0.86	0.03	0.34	25.45 ± 6.84	4.24 ± 0.64	3.33 ± 0.52	0.02	<0.001	0.83
CXCL10[3]	18.41 ± 4.55	17.90 ± 4.26	2.91 ± 0.55	1.00	0.01	0.01	6.44 ± 1.29	8.07 ± 0.99	5.17 ± 1.40	0.99	0.62	0.29
HP[2]	241.31 ± 215.68	59.36 ± 19.47	10.18 ± 2.56	1.00	0.47	0.48	2777.86 ± 1219.88	7.08 ± 1.62	14.73 ± 4.87	0.03	0.11	1.00
IL10(a)[1]	9.10 ± 1.33	18.15 ± 3.56	3.40 ± 0.54	0.92	0.05	0.003	6.56 ± 0.87	11.37 ± 2.18	4.01 ± 1.00	0.99	0.17	0.053
IL18[1]	5.35 ± 1.18	9.78 ± 2.30	3.00 ± 0.59	0.98	0.19	0.04	17.65 ± 2.41	14.21 ± 2.55	6.68 ± 0.79	0.96	0.07	0.35
IL1RN[1]	23.60 ± 5.07	68.60 ± 11.30	30.92 ± 7.18	0.15	1.00	0.16	178.33 ± 36.97	511.44 ± 149.53	68.19 ± 14.99	0.34	0.25	0.002
IL6(a)[1]	3.37 ± 0.64	2.36 ± 0.35	2.49 ± 0.23	0.84	0.95	1.00	6.40 ± 1.34	5.13 ± 0.58	3.06 ± 0.56	1.00	0.47	0.078
IL6(b)[1]	3.66 ± 0.56	2.38 ± 0.37	2.50 ± 0.35	0.70	0.81	1.00	8.36 ± 1.62	5.57 ± 0.74	3.26 ± 0.72	0.84	0.17	0.81
ITIH4(a)[2]	3.98 ± 0.89	2.45 ± 0.27	2.63 ± 0.37	0.70	0.51	1.00	12.50 ± 4.26	1.46 ± 0.21	3.79 ± 0.66	0.01	0.95	0.10
ITIH4(b)[2]	3.85 ± 1.03	3.31 ± 0.25	4.35 ± 0.55	0.96	1.00	0.99	13.74 ± 4.81	2.54 ± 0.58	5.67 ± 0.88	0.005	0.31	0.45
LBP[2]	197.03 ± 85.54	65.79 ± 16.09	85.56 ± 9.09	0.55	0.74	1.00	1389.84 ± 612.95	10.65 ± 4.65	42.33 ± 15.15	<0.001	0.051	0.04
NFKB1[4]	2.19 ± 0.13	2.98 ± 0.22	1.54 ± 0.11	0.46	0.004	<0.001	2.40 ± 0.18	2.78 ± 0.18	1.74 ± 0.12	0.77	0.07	0.002
NFKBIA[4]	2.31 ± 0.21	2.69 ± 0.24	1.45 ± 0.16	1.00	0.03	0.008	2.63 ± 0.33	3.56 ± 0.55	1.27 ± 0.11	0.73	0.01	<0.001
PAFAH1B1[4]	1.71 ± 0.08	1.64 ± 0.06	1.32 ± 0.08	0.85	0.004	0.08	1.79 ± 0.08	1.43 ± 0.06	1.40 ± 0.04	0.20	0.13	1.00
PDB-2[4]	311.91 ± 107.09	351.50 ± 58.67	906.21 ± 381.26	1.00	0.83	0.86	143.78 ± 13.98	179.47 ± 40.83	105.94 ± 20.08	0.95	0.96	1.00
SAA[2]	14.01 ± 5.49	9.21 ± 1.89	4.60 ± 1.37	1.00	0.49	0.30	38.64 ± 14.11	4.42 ± 0.38	4.13 ± 0.78	0.04	0.06	1.00
TF[2]	62.37 ± 56.27	63.68 ± 9.22	12.55 ± 3.48	0.85	0.99	0.46	409.07 ± 237.01	29.65 ± 11.58	8.17 ± 1.09	0.75	0.13	0.82
TGFB1[1]	2.87 ± 0.20	5.86 ± 0.54	1.77 ± 0.20	<0.001	0.001	<0.001	4.27 ± 0.29	6.81 ± 0.72	2.49 ± 0.19	0.03	0.004	<0.001
TLR4[4]	1.63 ± 0.11	3.74 ± 0.22	2.14 ± 0.10	0.02	1.00	0.02	3.02 ± 0.52	5.08 ± 0.72	2.63 ± 0.24	0.07	1.00	0.03
TNF[1]	61.12 ± 13.59	118.16 ± 24.56	35.74 ± 5.73	0.18	1.00	0.10	75.63 ± 14.26	109.03 ± 22.78	53.10 ± 9.55	0.97	0.97	0.64
TNFAIP3[1]	2.02 ± 0.31	2.67 ± 0.15	1.53 ± 0.14	0.49	0.81	0.05	2.92 ± 0.46	3.40 ± 0.77	1.75 ± 0.04	1.00	0.34	0.15

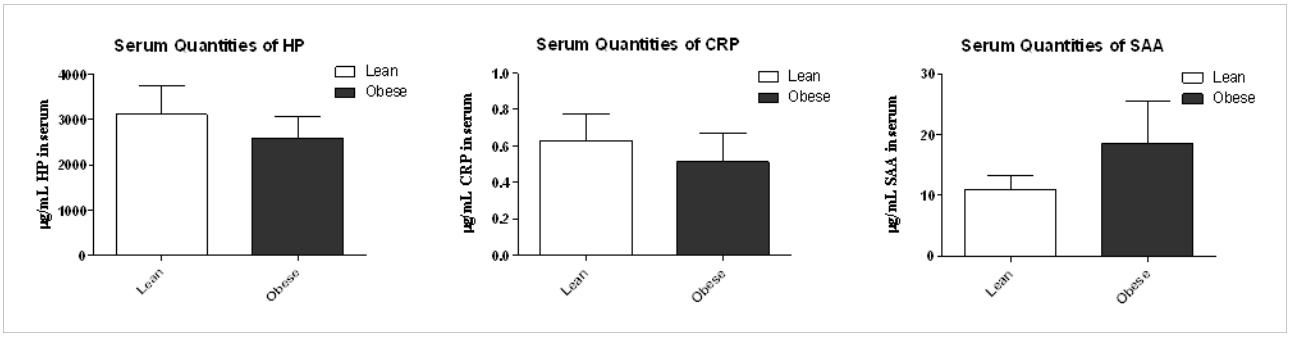
a means significantly difference between RPAT and VAT. b means significantly difference between RPAT and neck SAT. c means significantly difference between VAT and neck SAT. 1 is cytokines. 2 is acute phase proteins. 3 is chemokines. 4 is immune regulatory genes. 5 is adipokines.

Serum Concentrations of Proteins

The serum concentrations of HP, CRP and SAA are depicted in Fig. 2. No significant differences in HP, CRP or SAA serum concentrations were found between the lean and obese Göttingen minipigs. The serum levels of TNF-α and IL-6 were below the detection limit (except in 1 lean minipig in the IL-6 ELISA) and are therefore not depicted. None of the transcripts coding for these proteins were significantly differentially regulated in the liver of obese compared to lean pigs, however, in the

VAT the APPs HP and SAA were significantly differentially down-regulated in the obese minipigs compared to the lean minipigs and IL6 was up-regulated in both the RPAT and the VAT in the obese minipigs compared to the lean (Fig. 1).

Figure 2: Serum protein concentrations



Serum concentrations of HP (A), CRP (B) and SAA (C) measured with ELISA, lean n=7 and obese n=7. Error bars depict SEM.

DISCUSSION

Obesity-related changes in the expression of innate immune system related genes and possible similarities to human obesity of obese Göttingen minipigs has to our knowledge not been thoroughly described before. The obese Göttingen minipigs had significantly higher bodyweights than the lean pigs (S. G. Moesgaard et al, unpublished data) and we find clear differences in the gene regulation in the obese minipigs compared to lean, including APP and cytokine genes in adipose tissues. The expression of a number of other relevant obesity-related genes in these pigs will be published elsewhere (S. Cirera et al, University of Copenhagen, Frederiksberg C, Denmark, personal communication).

Interestingly, three genes were significantly differentially regulated in all three tissues of the obese pigs as compared to the lean pigs in all three adipose tissues, namely CCL3L1, IL1RN and CD200. CCL3L1, a chemokine also known as macrophage inflammatory protein 1-α (MIP-1α), is up-

304 regulated in the obese minipigs and is involved in acute inflammation by recruiting and activating
305 polymorphonuclear leukocytes (Wolpe et al., 1989). Previous work in mice has established that this
306 gene is up-regulated in the epididymal white adipose tissue from obese mice and may play a role in
307 the development of type II diabetes (Xu et al., 2003). To our knowledge the gene expression of
308 CCL3L1 has not yet been investigated in human adipose tissues, however, the serum protein
309 concentration is similar in lean and obese humans (Kim et al., 2006). The up-regulation of this pro-
310 inflammatory gene (CCL3L1) is accompanied by the down-regulation of the anti-inflammatory
311 gene CD200. CD-200 is a receptor on the surface of e. g. macrophages and helps down-regulate
312 myeloid cell activity (Hoek et al., 2000; Wright et al., 2000). The combined effect of these changes
313 in the adipose tissues of the obese minipigs compared to the lean would be pro-inflammatory.
314 However, the most up-regulated gene in VAT and RPAT of obese pigs is IL1RN. The product of
315 IL1RN is the interleukin 1 receptor antagonist (IL1ra), a naturally occurring inhibitor of the pro-
316 inflammatory proteins IL-1α and –β (Seckinger et al., 1987). White adipose tissue has been found to
317 be a major source of I IL1ra in both mice and men (Juge-Aubry et al., 2003) and IL1ra may play a
318 role in acquired resistance to leptin in obesity in rats and humans (Luheshi et al., 1999; Meier et al.,
319 2002). IL1RN is significantly up-regulated in the VAT compared to neck SAT in the obese pigs,
320 which could counteract the pro-inflammatory effects of the down-regulated CD200 and the up-
321 regulated CCL3L1, and thereby offer an explanation as to why the cytokine and APP serum protein
322 concentrations is not elevated by obesity. Interestingly, IL6 is up-regulated in the RPAT and VAT
323 in the obese minipigs, and previous reports suggest that IL-6 suppresses inflammation via induction
324 of IL1ra synthesis in humans (Tilg et al., 1994). This is supported by the findings that the up-
325 regulation of IL1RN expression in the RPAT and VAT, where IL6 is up-regulated, is much higher
326 than in the neck SAT (7.5 times higher in the RPAT and VAT of the obese pigs, and only 2 times
327 higher in the neck SAT of the obese minipigs compared to lean).

A correlation between increased adipose tissue expression and elevated serum levels of various innate immune factors in obesity has been established in humans for a number of proteins, including IL-6 (Fontana et al., 2007), haptoglobin (Chiellini et al., 2004) and SAA (Poitou et al., 2006). However, such correlations were not found in the Göttingen minipigs investigated in the present study. Of the three genes mentioned, adipose tissue expression of only IL6 was elevated, in both the RPAT and the VAT, however, no increases in serum concentration of IL-6 were measured in the Göttingen minipigs. The expression of both SAA and HP was down-regulated in the VAT and no elevated serum levels of these proteins were detected either. This contrasts with the serum protein changes observed in the human response to obesity, in which CRP, SAA and haptoglobin serum concentrations are elevated (Chiellini et al., 2004; Esposito et al., 2003; van Dielen et al., 2001). A reason for this could be that the fold changes of the genes significantly affected by obesity in the liver are very small, and perhaps too small for the changes to be translated into elevated serum protein concentration. Another reason why the Göttingen minipigs in this study do not show a human-like serum obesity response could be that their diet contained no excess fat or sugar, and it is possible that a standard diet does not induce a systemic or local low-grade inflammation. However, the pigs have been obese for a prolonged period of time, as they are euthanized at 41-47 mo, which mimics human obesity.

Preliminary results in young Göttingen minipigs indicate that 3-4 mo of high calorie, high cholesterol feeding leads to significant changes in acute phase protein serum concentrations (T. P. Ludvigsen et al., Novo Nordisk A/S, Maaløv, Denmark, personal communication), however, further investigation is needed to establish whether this is due to age- or diet differences. Therefore it could be interesting to repeat the investigations in pigs fed a high calorie diet for the same extended period of time (41-47 mo) to see if both the gene expression and serum concentrations of several innate immune factors are affected by obesity when fed a high calorie diet. Furthermore, it could be

interesting to investigate whether the changes in gene expression in obesity is translated into measurable changes in the local expression of corresponding proteins in the tissues in question.

In summary, the gene expression of Göttingen minipig is affected by obesity, as a wide range of innate immunology related genes are significantly differentially regulated in the obese pigs compared to the lean pigs. However, in contrast to human obesity, this does not translate into changes in serum concentrations of cytokines and APPs in the obese pigs compared to the lean.

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4.4 Results for Study II – Ossabaw minipigs (unpublished)

The Ossabaw minipigs are widely accepted as good model for human obesity, however, no studies into the response of the innate immune response to obesity have been made on these pigs. Therefore, this study into the expression of innate immune factors in liver, two adipose tissues and blood was conducted. Tissues from 12 pigs, and serum from 10 pigs, were used (n=7 for the two adipose tissues) and all pigs were female. The obese pigs weighed significantly more than the lean (p=0.008) and further descriptions regarding the pigs is shown in Table 10. The pre-PCR data for the 12 pigs and three tissues as measured with the NanoDrop and Bioanalyzer (discussed in section 2.1.1) is shown in Table 7. Even though the yields (ng/µl) were high in all tissues, and the 260/230 and 260/280 ratios and RINs of the liver showed RNA of high quality, the 260/230 ratios were generally low for the adipose tissues. The 260/280 ratios were within the accepted range of 1.8-2 for all tissues, however, the average RINS for the adipose tissues were low (below 5), and all the samples in the VAT had consistently low RINs.

Tissue	ng/µl	260/230	260/280	RIN
Liver	393-3166 (1931)	1.95-2.27 (2.18)	2.01-2.16 (2.11)	7.1-8.5 (7.7)
Abdominal SAT	126-922 (463)	0.61-1.99 (1.64)	1.88-2.03 (1.94)	2.4-6.8 (4.6)
VAT	56-871 (269)	0.30-2.10 (1.36)	1.78-1.93 (1.86)	2.3-3.4 (2.7)

Table 7: Yields, ratios and RINs of the Ossabaw minipigs in the three measured tissues. The lowest and highest values are shown, with the mean in parenthesis. n=12 for liver, n=7 for abdominal SAT and VAT.

cDNA synthesis of the samples was performed in duplicates using 500 ng of total RNA. Primers were designed as described in (Skovgaard *et al.* 2009), and a table of the primers used for this study is shown in Paper III p. 70. The expression of 48 innate immune response genes, including five putative reference genes, was tested in a high-throughput chip-based qPCR (Fluidigm). Expression data was acquired using the Fluidigm Real-Time PCR Analysis Software 3.0.2 (Fluidigm). For preprocessing of data, normalization and relative quantification GenEx5 (MultiD, Göteborg, Sweden) was used. GeNorm was used to select the most stable reference genes for normalization; for the adipose tissues *GAPDH* was chosen, and for liver *GAPDH*, *B2M* and *RPL13A* were chosen. Cq-values were calculated relative to the least expressed sample for each primer set and data was log2 transformed to approach normal distribution prior to statistical analysis with either a two-tailed, unpaired *t*-test or a one-way ANOVA with Tukey-Kramer post test. Statistics were performed in GenEx5 or Prism5 (GraphPad Software Inc.). Changes in gene expression were considered significant if $p \leq 0.05$ and biologically relevant if the fold change was at least ±1.5. Data was evaluated for outliers with Grubbs outlier test (Grubbs 1969) where applicable. Data is expressed as the mean ± SEM.

Contamination and primer specificity were tested with –RT samples and a NTC (data not shown). NTC showed no contamination, neither did the –RT sample for liver, but for the adipose tissues the –RT sample revealed genomic DNA contamination/low primer specificity in 24 of the 48 primers tested. As the Ossabaw data was not purposed for publication, due to small groups and low quality of the RNA, the genes showing contamination were not removed before preprocessing of data.

In the preprocessing of data, several genes were removed due to low efficiency or in the quality validation of the technical replicates (cDNA) if the standard deviation was above 15% (as described in appendix 9.2): For both tissues *CRP*, *IL1B*, *COX2*, *IL6(b)*, *ITIH4(b)*, *IL18*, *ORM1(a)*, *IL10(b)*, *IFNG*, *TNF*, and *AOAH* were

removed, in the liver additionally *ADIPOQ*, *IL6(a)*, *SFTPA1*, *IL8(a)*, *CD36* and *ORM1(b)* were removed and in the adipose tissues additionally *CD40*, *PBD2*, *FIB*, *TLR4*, *CXCL10* and *CCL3* were removed. Furthermore, due to large differences between the two technical replicates of cDNA (>30%) two VAT samples and one abdominal SAT sample were removed (all samples from the lean group). Because of this, the lean group in the VAT only consisted of two pigs, and it was not possible to perform statistical analysis on the data. The expression of the remaining samples, and remaining 26 genes in the liver and 25 genes in the adipose tissues are shown in Table 8.

Interestingly, as seen in Table 8, all the genes showing significant differential expression in the liver are downregulated in the obese pigs. No clear patterns are evident as the genes being downregulated are different types of innate immune genes (chemokines, immune regulatory and APPs), and are involved in both the proinflammatory response (*CCL2*, *CD14*, *FIB* and *HP*) and in the anti-inflammatory response (*IL1RN* and *TF*). In the abdominal SAT only onw gene is significantly differentially regulated, namely *LBP*, an APP part of the proinflammatory response, which is upregulated 9-fold in the obese pigs.

	Liver		Abdominal SAT		VAT	
	Lean	Obese	Lean	Obese	Lean	Obese
ADIPOQ	NQ	NQ	1 ± 0.94	0.82 ± 0.25	1 ± 0.07	1.50 ± 0.27
APOA1	1 ± 0.11	1.52 ± 0.39	1 ± 0.56	16.30 ± 15.53	1 ± 0.18	5.33 ± 1.41
C3	1 ± 0.11	0.80 ± 0.17	1 ± 0.11	9.65 ± 3.54	1 ± 0.55	57.87 ± 32.28
CCL2	1 ± 0.31	0.23 ± 0.08*	1 ± 0.20	14.61 ± 8.64	1 ± 0.93	2.47 ± 1.34
CCL3L1	1 ± 0.36	0.63 ± 0.30	NQ	NQ	NQ	NQ
CCL5	1 ± 0.10	0.78 ± 0.18	1 ± 0.53	3.61 ± 1.67	1 ± 0.38	4.00 ± 1.33
CD14	1 ± 0.12	0.50 ± 0.10**	1 ± 0.59	2.50 ± 0.75	1 ± 0.59	9.80 ± 8.07
CD163	1 ± 0.06	2.25 ± 0.71	1 ± 0.31	0.77 ± 0.24	1 ± 0.16	4.92 ± 3.28
CD200	1 ± 0.12	1.00 ± 0.49	1 ± 0.40	1.75 ± 0.80	1 ± 0.67	6.90 ± 6.53
CD36	NQ	NQ	1 ± 0.73	0.72 ± 0.22	1 ± 0.20	1.50 ± 0.40
CD40	1 ± 0.18	0.64 ± 0.14	NQ	NQ	NQ	NQ
CXCL10	1 ± 0.33	0.38 ± 0.12	NQ	NQ	NQ	NQ
FIB	1 ± 0.06	0.55 ± 0.11**	NQ	NQ	NQ	NQ
HP	1 ± 0.05	0.57 ± 0.07***	1 ± 0.44	4.05 ± 3.39	1 ± 0.06	18.99 ± 13.14
IL10(a)	1 ± 0.20	0.84 ± 0.42	1 ± 0.33	1.15 ± 0.32	1 ± 0.91	10.71 ± 10.03
IL1RN	1 ± 0.21	0.18 ± 0.12**	1 ± 0.05	1.30 ± 0.42	1 ± 0.88	0.42 ± 0.24
IL6(a)	NQ	NQ	1 ± 0.13	9.79 ± 7.75	1 ± 0.82	1.33 ± 0.66
IL8(a)	NQ	NQ	1 ± 0.38	3.16 ± 1.83	1 ± 0.88	4.50 ± 3.52
IL8(b)	1 ± 0.39	0.65 ± 0.36	1 ± 0.32	5.99 ± 3.99	1 ± 0.93	2.71 ± 2.03
ITIH4(a)	1 ± 0.13	0.57 ± 0.21	1 ± 0.68	1.14 ± 0.51	1 ± 0.23	22.69 ± 18.16
LBP	1 ± 0.13	1.05 ± 0.46	1 ± 0.35	9.60 ± 2.92*	1 ± 0.78	40.08 ± 37.22
NFKB1	1 ± 0.07	0.75 ± 0.16	1 ± 0.40	1.65 ± 0.69	1 ± 0.18	2.09 ± 0.74
NFKBIA	1 ± 0.30	0.53 ± 0.06	1 ± 0.31	2.51 ± 1.37	1 ± 0.11	5.06 ± 2.76
ORM1(b)	NQ	NQ	1 ± 1.00	97.89 ± 97.85	1 ± 0.73	110.78 ± 102.74
PAFAH1B1	1 ± 0.05	0.87 ± 0.05	1 ± 0.32	0.99 ± 0.33	1 ± 0.15	1.92 ± 0.35
PDB2	1 ± 0.23	1.06 ± 0.45	NQ	NQ	NQ	NQ
SAA	1 ± 0.27	0.59 ± 0.51	1 ± 0.38	3.76 ± 1.77	1 ± 0.90	8.02 ± 7.62
TF	1 ± 0.11	0.62 ± 0.11*	1 ± 0.99	15.59 ± 15.51	1 ± 0.85	37.07 ± 31.72
TGFB1	1 ± 0.13	0.88 ± 0.18	1 ± 0.32	1.43 ± 0.54	1 ± 0.23	4.96 ± 3.58
TLR4	1 ± 0.09	1.64 ± 0.59	NQ	NQ	NQ	NQ
TNFAIP3	1 ± 0.61	0.72 ± 0.45	1 ± 0.12	2.44 ± 1.23	1 ± 0.42	5.61 ± 3.93

Table 8: Relative gene expression (mRNA levels) of innate immune genes in lean (set to 1) and obese Ossabaw minipigs in liver and thre types of adipose tissue as analyzed by qPCR. For liver; lean n=7, obese n=5, for abdominal SAT; lean n=3, obese n=3, for VAT; lean n=2, obese n=3. (*)$p \leq 0.05$, (**)$p < 0.01$, (***)$p < 0.001$. NQ, not quantifiable.

The concentrations of serum proteins HP, CRP, IL-6, TNF-α and SAA were determined using sandwich ELISAs. To determine IL-6, TNF-α and SAA commercially available ELISAs were used according to manufacturer's protocols, and to determine CRP and HP in-house assays were used (for details on all ELISAs, see Paper I p. 27). Samples were run in triplicates to eliminate pipetting variation, and dilutions of a standard of known concentration were present in all ELISAs. Significance was tested with a two-tailed, unpaired *t*-test and $p \leq 0.05$ was considered significant. Data was evaluated for outliers with Grubbs outlier test where applicable. Serum protein expression for lean and obese Ossabaw minipigs is shown in Figure 12. Protein concentrations of TNF-α and for most samples of IL-6 were below detection level. No significant difference was found in any of the 5 measured serum proteins.

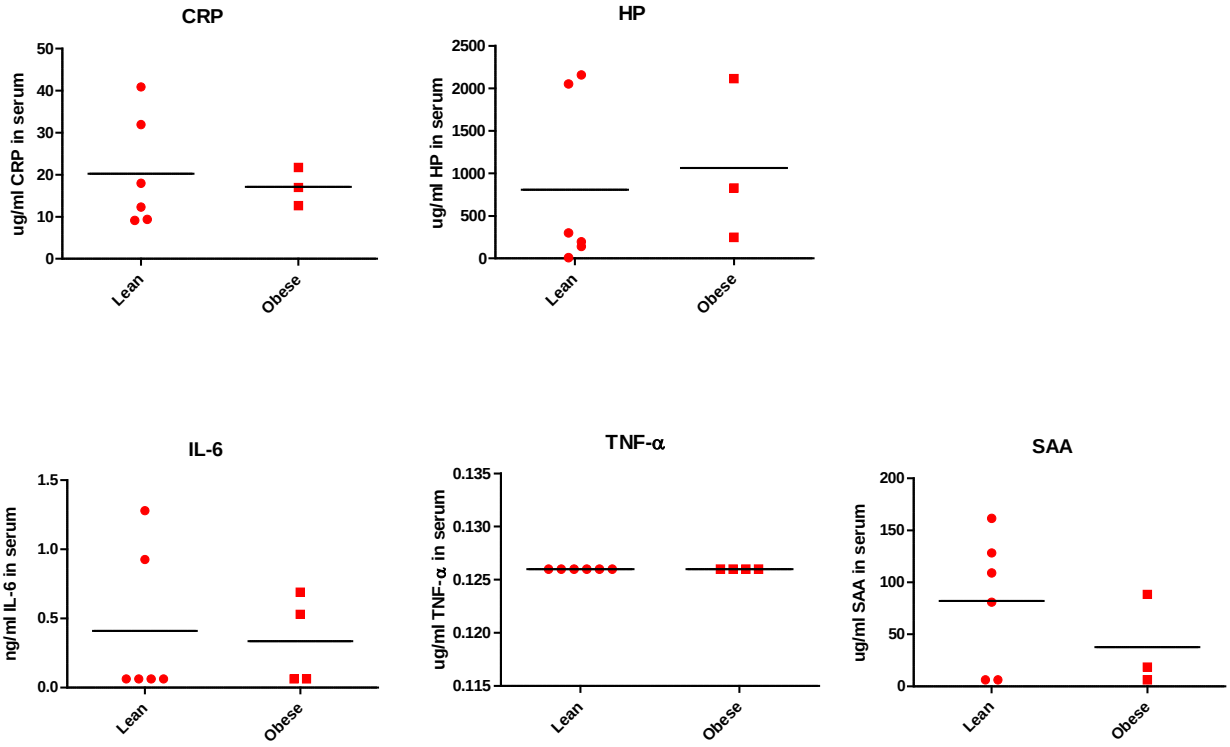

Figure 12: Scatter plot of serum concentrations of CRP, HP, IL-6, TNF-α and SAA measured with ELISA in lean (n=6) and obese (n=4 for IL-6 and TNF-α, n=3 for CRP, HP and SAA).

5 Study III: Comparison of the three breeds of porcine obesity models

All three breeds of pigs investigated in this thesis have been used as in studies as models for human metabolic perturbations. Therefore, it would be interesting to compare the three breeds, despite age and feed differences.

5.1 Background

The domestic pigs were 8-9 months of age, the Göttingen minipigs 41-47 months of age and the Ossabaw minipigs were 14-16 months of age at euthanasia, making the pigs very different in age. Furthermore, the diets of the three breeds of pigs also differed in the contents of protein, fat and carbohydrates, as seen in Table 9. The compositions of the feeds of the Göttingen minipigs were low fat/high carbohydrates as both were standard diets for minipigs (Altromin 9023 and 9033). The diet of the obese Ossabaw minipigs contained most fat (46%) of all the diets. The diets are described in detail in appendix 9.11.

	Domestic pigs	**Göttingen minipigs**		**Ossabaw minipigs**	
%	Lean/obese	Lean	Obese	Lean	Obese
Protein	18	19	25	18.5	8
Fat	25	8	11	10.5	46
Carbohydrates	57	73	64	71	43

Table 9: Composition as total content (%) of the experimental diets of the three breeds of pigs

The literature comparing the three breeds is very limited. No papers have been published, to our knowledge, comparing the Ossabaw and Göttingen minipigs directly. In the 1970s and 80s a number of papers were published comparing the Ossabaw minipig with Yorkshire pigs to describe the Ossabaw minipigs as an obese phenotype pig. Some of these are referred to in Section 4.1.1.2. Göttingen minipigs have also been compared to domestic pigs; to establish that the usability of Göttingen minipigs as a model for chronic heart failure and myocardial infarction was higher than Yorkshire pigs (Schuleri *et al.* 2008), and to establish that Göttingen minipigs were better models for atherosclerosis than Swedish Landrace pigs (Jacobsson 1986).

5.1.1 The acute phase protein orosomucoid (ORM)

Orosomucoid (ORM), also known as α-1-acid glycoprotein, is a member of the APP family. During acute infection the serum concentration of ORM increases 2-5 times in all mammals, except in the pig where ORM is a negative APP (Skovgaard *et al.* 2009). It is one of the most abundant plasma proteins, as it accounts for approximately 1% of all plasma proteins (Hochepied *et al.* 2003; Fournier *et al.* 2000). In humans two variants of ORM exists, ORM1 and ORM2, which are coded by two different genes, and the major component of serum ORM is ORM1 (Fournier *et al.* 2000). In pigs an *ORM* gene has been found, which shares 70% homology with the coding sequence of human *ORM1* (Stone & Maurer 1987; Fournier *et al.* 2000). ORM has been found to have anti-inflammatory and immunemodulatory effects (Hochepied *et al.* 2003; Williams *et al.* 1997) and ORM has been implicated in maintenance of metabolic homeostasis in obesity by suppressing local and systemic inflammation in mice (Lee *et al.* 2010). The mRNA expression of *ORM* in adipose tissues of obese mice were elevated with increasing ORM plasma levels (Lee *et al.* 2010), however, this relationship was not found in humans (Alfadda *et al.* 2012). In humans, correlations were found between circulating ORM levels, BMI and body fat percent (Alfadda *et al.* 2012) indicating that

secretion of ORM from adipose tissues may be a contributing source for the rise of circulating ORM levels in the blood in obesity. To our knowledge, no information of the differential expression of ORM in adipose tissues between lean and obese humans or pigs has been published. Furthermore, even though ORM is a negative APP in acute infections in pigs, a proteomics study in Ossabaw minipigs found that serum ORM levels were elevated in Ossabaws fed an atherogenic diet compared to control pigs (Bell *et al.* 2010), indicating that the response of ORM in pigs to obesity is similar to the human response.

Due to this, and to interesting data from the primer set of *ORM1* on the high-throughput qPCR of the three breeds where the expression of *ORM1* was too high to be analyzed in the domestic pigs and too low to be analyzed in the minipigs, we decided to investigate the expression of this interesting APP in lean and obese domestic pigs, Göttingen minipigs and Ossabaw minipigs.

5.2 Hypothesis for Study III

- The response of innate immune genes and serum proteins to obesity is differentiated between the domestic pigs and the two breeds of minipigs, with the response of the minipigs being more human-like
- ORM serum concentrations are higher in obese pigs than lean, especially in the Göttingen and Ossabaw minipigs
- The expression of the *ORM1* gene is upregulated in obese pigs compared to lean

5.3 Results of Study III

5.3.1 Phenotypic parameters in the three breeds of pig

Parameters	Domestic pig			Göttingen minipigs			Ossabaw minipigs		
	Lean (n=9)	**Obese** (n=10)	***p*-value**	**Lean** (n=7)	**Obese** (n=7)	***p*-value**	**Lean** (n=4)	**Obese** (n=3)	***p*-value**
Body weight (kg)	119.1±3.2	170.1±4.9	0.03	50.3±1.6	92.6±5.2	<0.0001	60.4±7.6	100.00±3.3	0.008
Fasting blood glucose (mmol/L)	4.5±0.1	4.4±0.2	0.59	3.8±0.1	3.7 ±0.4	0.87	4.2±0.1	4.6±0.2	0.16
Fasting insulin (pmol/L)	12.1±3.4	29.3±5.2	0.87	141.2±32.4	39.2±9.9	0.01	15.0±3.5	34.7±7.5	0.10
Total cholesterol (mmol/L)	2.1±0.1	3.2±0.1	0.01	1.8±0.1	2.0±0.1	0.17	2.0±0.1	12.8±2.0	0.03
Triglycerides (mmol/L)	0.3±0.03	0.4±0.05	0.85	0.3±0.04	0.5±0.07	0.07	0.3± 0.03	0.5 ±0.03	0.02

Table 10: Summary of weights and blood parameters in the three breeds of pigs. Domestic pig data is taken from (Christensen *et al.* 2012), data from Göttingen minipigs is from (Moesgaard et al, article in preparation) and data from Ossabaw minipigs is from (Lee *et al.* 2009). Data is expressed at mean±SEM.

The summary of several phenotypic traits relevant for animal models of obesity is shown in Table 10. None of the three breeds of pigs showed differential fasting blood glucose levels between lean and obese pigs. The domestic pigs and the Göttingen minipigs only showed differential levels of one parameter between lean and obese pigs, namely total cholesterol (p=0.01) and fasting insulin (p=0.01), respectively, whereas the Ossabaw minipigs showed differential levels in two parameters between lean and obese pigs, namely total cholesterol (p=0.03) and triglycerides (p=0.02).

5.3.2 Paper IV

Orosomucoid expression in liver, adipose tissues and serum of lean and obese domestic pigs, Göttingen minipigs and Ossabaw minipigs

Rødgaard T, Stagsted J, Christoffersen BØ, Cirera S, Moesgaard SG, Sturek M, Alloosh M, Heegaard PMH. Vet Immunol Immunopathol. 2012, short communication, in press

Orosomucoid is an APP with anti-inflammatory and immunomodulatory effects, which has been implicated in maintaining metabolic homeostasis in obesity. The mRNA and serum expression of ORM has been investigated in obese humans and mice, but not in pigs, even though porcine obesity models are widely used for obesity-related metabolic symptoms.

The objective was to characterize the ORM mRNA expression in liver and three types of adipose tissues; RPAT/abdominal SAT, VAT and neck SAT, as well as the serum concentrations of ORM in three breeds of porcine obesity models; domestic pigs, Göttingen minipigs and Ossabaw minipigs.

We found no changes in mRNA expression of *ORM* in any of the four investigated tissues in lean compared to obese pigs, or between the adipose tissues of obese pigs. Ossabaw minipigs were the only breed to show a differentiated ORM serum response, with elevated concentrations of ORM in obese Ossabaw minipigs, which is similar to the human response. Therefore, in this regard, the obese Ossabaw minipigs behave more similar to obese humans.

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Short communication

Orosomucoid expression profiles in liver, adipose tissues and serum of lean and obese domestic pigs, Göttingen minipigs and Ossabaw minipigs

Tina Rødgaard[a], Jan Stagsted[b], Berit Ø. Christoffersen[c], Susanna Cirera[d], Sophia G. Moesgaard[d,1], Michael Sturek[e], Mouhamad Alloosh[e], Peter M.H. Heegaard[a,*]

[a] Innate Immunology Group, National Veterinary Institute, Technical University of Denmark, Frederiksberg C, Denmark
[b] Department of Food Science, Aarhus University, Tjele, Denmark
[c] Novo Nordisk A/S, Maaløv, Denmark
[d] Department of Veterinary Clinical and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg C, Denmark
[e] Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, IN, USA

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ABSTRACT

The acute phase protein orosomucoid (ORM) has anti-inflammatory and immunomodulatory effects, and may play an important role in the maintenance of metabolic homeostasis in obesity-induced low-grade inflammation. Even though the pig is a widely used model for obesity related metabolic symptoms, the expression of ORM has not yet been characterized in such pig models. The objective of this study was to investigate the expression of ORM1 mRNA in liver, visceral adipose tissue, subcutaneous adipose tissue (SAT) from the abdomen or retroperitoneal abdominal adipose tissue and SAT from the neck, as well as the serum concentration of ORM protein in three porcine obesity models; the domestic pig, Göttingen minipigs and Ossabaw minipigs.

No changes in *ORM1* mRNA expression were observed in obese pigs compared to lean pigs in the four types of tissues. However, obese Ossabaw minipigs, but none of the other breeds, showed significantly elevated ORM serum concentrations compared to their lean counterparts. Studies in humans have shown that the expression of ORM was unchanged in adipose tissue depots in obese humans with an increased serum concentration of ORM. Thus in this respect, obese Ossabaw minipigs behave more similarly to obese humans than the other two pig breeds investigated.

1. Introduction

Obesity is accompanied by low-grade systemic inflammation, which may contribute to development of obesity-associated metabolic abnormalities, such as atherosclerosis, type 2 diabetes and cardiovascular diseases (Bastard et al., 2006; Federico et al., 2010; Gil et al., 2007). A number of immune factors are involved in this low-grade inflammation and several of these factors are produced in the adipose tissues as well as other tissues (reviewed in for example Fain, 2010; Fantuzzi, 2005). A well-regulated acute inflammatory response is a self-limiting and localized non-adaptive response to tissue destruction/perturbation, whether it is of infectious or of aseptic origin. In contrast, the inflammatory response seen in obesity is systemic and chronic with few acute clinical manifestations, being rather characterized by a slightly

* Corresponding author at: Innate Immunology Group, DTU Veterinary, Bülowsvej 27, Building B, 1870 Frederiksberg C, Denmark.
Tel.: +45 35886241; fax: +45 35886001.
E-mail address: pmhh@vet.dtu.dk (P.M.H. Heegaard).
[1] Present address: Novo Nordisk A/S, Maaløv, Denmark.

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perturbed inflammation marker serum profile and having no easily definable targets (Fain, 2010). Prolonged and exaggerated inflammation is known to be detrimental to the host tissue and several studies have shown that even small changes in the levels of inflammation markers such as acute phase proteins (APPs) are associated with serious long-term complications (Ebeling et al., 1999; Ridker et al., 1998, 2000).

Orosomucoid (ORM), also known as alpha-1 acid-glycoprotein, is a member of the APP family and is synthesized mainly hepatically, however, extrahepatic synthesis has been reported including in adipose tissue (Fournier et al., 2000). ORM has anti-inflammatory and immunomodulatory properties, including anti-neutrophil and anti-complement activity functions (Hochepied et al., 2003; Williams et al., 1997). Humans have two isoforms ORM1 and ORM2 and pigs have one isoform (Fournier et al., 2000; Stone and Maurer, 1987). ORM is induced in response to metabolic and inflammatory signals in the adipose tissue of several types of obese mice accompanied by an increase in plasma concentration (Lee et al., 2010). Serum ORM concentrations in obese humans are significantly higher than those seen in lean individuals (Alfadda et al., 2012; Lee et al., 2010), however, no relationship between ORM1 expression in adipose tissues and ORM serum levels of obese humans could be demonstrated. In addition no differential expression in adipose tissues in obese humans was found (Alfadda et al., 2012). We have been unable to identify any published data examining the mRNA expression of *ORM* in liver and adipose tissues between lean and obese humans. The presence of ORM1 in adipose tissues may help maintain metabolic homeostasis by suppressing local and systemic inflammation (Lee et al., 2010). Interestingly, evidence suggests that porcine ORM is a negative APP (Skovgaard et al., 2009), as opposed to all other investigated mammals. The expression of ORM in obese pigs has not been thoroughly investigated, even though the pig is growing in popularity as a model animal for obesity (Litten-Brown et al., 2010), however, a proteomic analysis of blood serum in lean and obese Ossabaw pigs on an atherogenic diet demonstrated increased serum concentrations of ORM in the obese pigs (Bell et al., 2010).

In this study we investigated ORM gene expression in liver and adipose tissues, as well as ORM serum protein concentrations in three types of lean and obese pigs; the domestic pig, the Ossabaw and the Göttingen minipig. As both the Ossabaw and Göttingen minipigs have been proposed to be very good model animals for human obesity (Bellinger et al., 2006; Litten-Brown et al., 2010), they are expected to have a similar ORM response as humans, i.e. no differential ORM1 expression in adipose tissues of obese pigs and raised ORM serum levels in obese pigs compared to lean (Alfadda et al., 2012; Lee et al., 2010).

2. Materials and methods

2.1. Animals, diets and sampling

2.1.1. Domestic pig (Danish landrace/Yorkshire)

All experimental procedures involving domestic pigs (intercross containing 75% Danish Landrace:25% Yorkshire) were approved by the Danish Animal Experiments Inspectorate. They were all female and reared and housed in the same experimental stables of Aarhus University (Tjele, Denmark). At three months of age the pigs were fed a high-energy diet (containing 10% sugar and 10% soy oil, as described by Christensen et al. (2012)) ad libitum (obese, $n = 10$) or at 60% intake (lean, $n = 9$). At 8–9 months the pigs were euthanized and tissue samples from liver, abdominal s.c. adipose tissue (SAT) (a combination of deep and superficial SAT), mesenteric fat surrounding the appendix (VAT) and s.c. fat from the neck (neck SAT) were obtained and snap frozen in liquid nitrogen, and blood was collected for serum preparation. Tissue and serum samples were kept at −80 °C until analysis.

2.1.2. Göttingen minipigs

All experimental procedures involving Göttingen minipigs were approved by the Danish Animal Experiments Inspectorate. Female, ovariectomized Göttingen minipigs (Ellegaard Göttingen Minipigs A/S (Dalmose, Denmark)) were housed at the University of Copenhagen (Taastrup, Denmark). The obese pigs had previously been used in pharmacological studies with therapeutic peptides, but had been subjected to a suitable wash-out period prior to this study. Lean pigs ($n = 7$) were fed minipig standard chow; Altromin 9023 2× 150 g a day and obese pigs ($n = 7$) were fed minipig standard chow; Altromin 9033 ad libitum. The minipigs were euthanized at 41–47 months of age with pentobarbital and tissues were collected from the liver, neck SAT, cranioventral retroperitoneal abdominal adipose tissue (RPAT) and VAT and snap frozen in liquid nitrogen, and blood was collected for serum preparation. Tissue and serum samples were kept at −80 °C until analysis.

2.1.3. Ossabaw minipigs

All experimental procedures involving Ossabaw minipigs were approved by the Indiana University Animal Care and Use Committee and complied fully with the Guide for the Care and Use of Laboratory Animals and the American Veterinary Medical Association Panel on Euthanasia. Female Ossabaw miniature swine were obtained from the Indiana University School of Medicine and Purdue University breeding colony (West Lafayette, IN, USA). At the age of 6 months, pigs were divided into two groups and fed either a lean standard chow diet of 2200 kcal per day with 10.5% of the total energy derived from fat (lean, $n = 7$) or an excess calorie fructose and fat atherogenic diet of 4500–6000 kcal per day with 43% of the total energy derived from fat (obese, $n = 5$) for 30–40 weeks (Lee et al., 2009). At the end of the feeding programs animals were euthanized by cardiectomy under anaesthesia induced by tiletamine-zolazepam (5 mg/kg) and xylazine (2.2 mg/kg) given intramuscularly. After euthanasia, samples of liver, abdominal SAT and VAT were collected and snap frozen. No neck SAT samples were collected from Ossabaw minipigs. Liver samples were retrieved from all animals while adipose tissue samples were obtained from 4 of the lean pigs and 3 of the obese pigs. Blood was collected for serum preparation. Serum and tissue samples were stored at −80 °C until analysis.

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2.2. RNA extraction

For all types of pigs liver RNA was extracted using RNeasy Lipid tissue Midi kit (Qiagen, Copenhagen, Denmark) according to the manufacturer's protocol and any DNA present was removed from the samples using on-column RNase-free DNase digestion (Qiagen).

Extraction of RNA from the adipose tissues of the lean domestic pigs was done as described in (Rødgaard et al., 2012), using approximately 1 g of tissue.

For four of the obese domestic pigs, abdominal SAT RNA was isolated by the column-based RNeasy Lipid tissue Midi kit (Qiagen) according to manufacturer's protocol and treated with on-column RNase-free DNase digestion (Qiagen). For the remaining 6 obese domestic pigs, and the VAT and neck SAT of all the obese domestic pigs, a similar method was used but without the columns. Briefly, approximately 1 g of adipose tissue was homogenized with QIAzol Lysis Reagent (Qiagen), and chloroform was added. After centrifugation, isopropanol was added to the upper, aqueous phase. After another centrifugation step the pellet was washed three times in ethanol and RNA was dissolved in RNase-free water.

For extraction of RNA from the adipose tissues of the Göttingen and Ossabaw minipigs, approximately 1/2 g of adipose tissue was homogenized with QIAzol Lysis Reagent and centrifuged. Chloroform was added followed by isopropanol after centrifugation. The pellet was washed 2 times in ethanol and RNA was dissolved in RNase-free water.

RNA yields were measured on a NanoDrop (Spectrophotometer ND-1000, NanoDrop Technologies Inc, USA) and the RNA integrity number (RIN) was determined by electrophoresis in the Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA), with the use of Agilent RNA 6000 Nano Kit (Agilent Technologies) according to the manufacturer's protocol. 500 ng of total RNA was reverse transcribed using the QuantiTECT Reverse Transcription kit (Qiagen), according to the manufacturer's instructions.

2.3. Quantitative real time PCR (qPCR)

qPCR was performed on a Rotor-Gene Q instrument (Qiagen) using SYBR Green Jumpstart Taq Readymix without $MgCl_2$ (Sigma, Brøndby, Denmark). Each sample was run in triplicates for the *ORM1* gene and for two reference genes (*GAPDH* and *RPL13A*) that were pre-selected based on previous experiences with the samples (data not shown) using GeNorm (Vandesompele et al., 2002). Non template controls (NTC) and samples without reverse transcriptase in the cDNA synthesis were included as negative controls. Primers were designed as described previously (Skovgaard et al., 2009). Primers are described in Table 1.

The following cycling parameters were used: 5 min at 95 °C followed by 40 of two-step cycles of 5 s at 95 °C and 10 s at 60 °C (for *ORM1*) or 62 °C (for *GAPDH* and *RPL13A*). Melting curves were generated after each run (from 50 °C to 99 °C, increasing 1 °C/5 s) to confirm amplification of a single PCR product. Expression data were acquired using the Rotor-Gene Q series software, v. 2.0.2 (Qiagen) and exported to GenEx5 (MultiD, Göteborg, Sweden).

2.4. Serum protein quantification with enzyme-linked immunosorbent assay (ELISA)

ORM was analyzed by a competitive catching ELISA in which a mouse monoclonal antibody specific for porcine ORM was used as the catching antibody in the coating layer (1.62, prepared in-house Heegaard et al., in preparation). This was followed by simultaneous incubation with sample and biotinylated ORM purified from a porcine serum pool. Pooled pig serum calibrated against a porcine ORM calibrator (Saikin Kagaku Institute Co. Ltd., Japan) was used as a standard. The detection limit of the assay was 50 μg/mL. Outliers were identified by Grubbs outlier test (Grubbs, 1969), and removed from the dataset.

2.5. Data analysis and statistics

qPCR data pre-processing was done using GenEx5. Data were corrected for differences in PCR efficiencies for each primer assay individually and the geometric mean of the reference genes was used to normalize all samples. Relative expression for all samples was calculated relative to the least expressed samples for each primer assay. Gene expression data were log2 transformed in order to fit a normal distribution prior to one-way ANOVA with Tukey–Kramer posttest. Statistics were performed in GraphPad Prism v.5.02 (GraphPad Software, California, USA). For ELISAs significance was tested using a two-tailed unpaired *t*-test. Changes were considered to be significant if $p \leq 0.05$. Data are expressed as the mean ± standard error of the mean (SEM).

3. Results and discussion

ORM has been implemented in maintaining metabolic homeostasis and suppressing obesity-induced low-grade inflammatory responses (Lee et al., 2010), therefore we studied the expression of ORM in lean and diet-induced obese pigs from three obesity relevant breeds. For all breeds, the mean body weight at the time of slaughter was significantly higher for the obese animals than for the lean animals (domestic pigs – 119.1 ± 3.2 (lean) vs. 170 ± 4.9 kg (obese), $p = 0.03$; Göttingen minipigs – 50.3 ± 1.6 (lean) vs. 92.6 ± 5.2 kg (obese), $p < 0.0001$; Ossabaw minipigs – 60.4 ± 7.6 (lean) vs. 100.0 ± 3.3 kg (obese), $p = 0.008$).

The Ossabaw minipig is the only breed in this study to show obesity-associated elevated serum levels of ORM (Fig. 1). Previous studies (Lee et al., 2009) have shown a high number of significant differences in blood parameters of obese pigs compared to lean pigs with significantly elevated levels of total cholesterol ($p = 0.03$) and triglycerides ($p = 0.02$) (and insulin, though not significantly, $p = 0.10$). According to Christensen et al. (2012) the only obesity feature affected in domestic pig is total cholesterol ($p = 0.01$) and in the Göttingen minipigs the only obesity feature affected by obesity was fasting insulin (Moesgaard et al., in preparation).

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Table 1
Primers used for qPCR.

Gene symbol	Gene name	Primer sequence	Amplicon length	$MgCl_2$	Run efficiency	R^2
ORM1	Orosomucoid 1	F: AGTCCTGAGCCTCCTTCCTC R: GCCGAGCCGATATAATACCA	123	3 mM	1.10	0.988
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F: ACCCAGAAGACTGTGGATGG R: AAGCAGGGATGATGTTCTGG	79	1.5 mM	1.08	0.992
RPL13A	Ribosomal protein L13a	F: ATTGTGGCCAAGCAGGTACT R: AATTGCCAGAAATGTTGATGC	76	3 mM	0.97	0.997

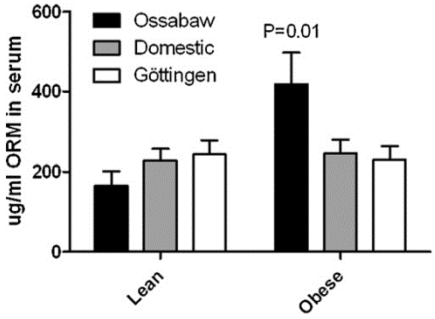

Fig. 1. Serum protein concentrations of ORM. Measured with ELISA. $n = 6$ for the lean Ossabaw pigs, $n = 4$ for the obese. $n = 7$ for lean and obese Göttingen minipigs, and $n = 9$ for the lean domestic pigs, $n = 10$ for the obese.

Fig. 2 shows the expression of ORM1 in the three pig breeds. Due to gene expression below detection level in some of the Ossabaw minipig samples, the number of samples from these groups became too small for the samples to be evaluated statistically (obese VAT $n = 2$, lean VAT $n = 2$, obese abdominal SAT $n = 2$, lean abdominal SAT $n = 1$). Domestic and Göttingen pig groups both showed a tendency towards a decreased relative expression of ORM1 in VAT of obese compared to lean pigs ($p = 0.16$ and $p = 0.12$, respectively) while in the two measurable Ossabaw VAT obese-lean sample pairs ORM1 expression was increased in the obese samples.

No changes in serum ORM concentration was observed in obese domestic pigs and obese Göttingen minipigs, however, obese Ossabaw minipigs showed a significant increase in the serum concentration of ORM compared to lean (Fig. 1). The ORM concentration in the lean group corresponded well with the baseline ORM concentration in non-obese, healthy Ossabaw pigs (unpublished).

No *ORM1* mRNA response correlating with the ORM serum response was found in this study, looking at *ORM1* responses in liver, abdominal SAT/RPAT, VAT and neck SAT, suggesting other sources of serum ORM. As previous studies have shown, other tissues and cells show ORM expression (Fournier et al., 2000), although not to the same level as the liver and adipose tissues (Lee et al., 2010). This is very different from the synthesis of ORM in a normal acute phase response during infection, where hepatic secretion is the main contributor to ORM in serum with no activity in the adipose tissues in several species (Fournier et al., 2000). Furthermore, it is remarkable that the response of ORM to obesity in pigs is similar to human response, with raised serum concentrations in obesity, as a previous study suggests that ORM is a negative APP in pigs during acute infection (Skovgaard et al., 2009). This fact only adds to the usability of the pig as a model for human obesity.

The Göttingen minipigs are older (41–47 months of age) than the Ossabaw minipigs (14–16 months of age) and the domestic pigs (8–9 months of age), and it is very possible that the innate immune features involved in systemic inflammation differ with age in pigs, as in humans (Gomez

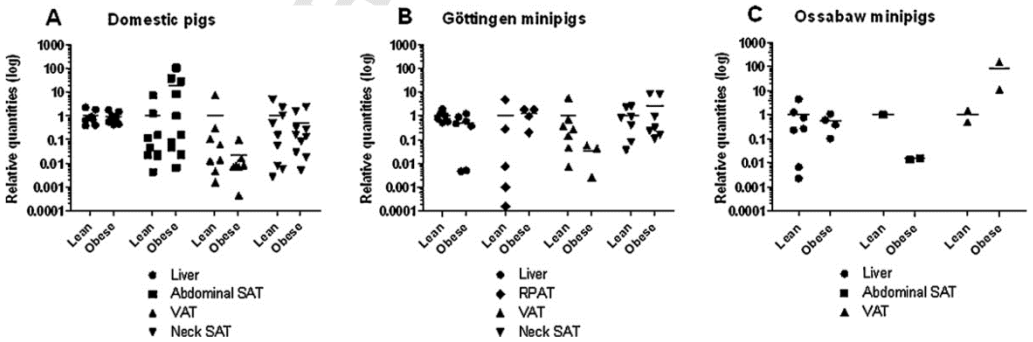

Fig. 2. Expression of ORM1 in domestic pig, Ossabaw and Göttingen minipigs in liver, abdominal SAT/RPAT, VAT and neck SAT as measured with qPCR. Relative to lean expression (set to 1). (A) Domestic pigs: Lean group – $n = 8$ (liver and VAT), $n = 9$ (abdominal and neck SAT). Obese group – $n = 9$ (liver), $n = 10$ (abdominal and neck SAT) and $n = 7$ (VAT). (B) Göttingen minipigs: Lean group – $n = 6$ (liver), $n = 5$ (RPAT) and $n = 7$ (VAT and neck SAT). Obese group: $n = 7$ (liver and neck SAT), $n = 4$ (RPAT) and $n = 3$ (VAT). (C) Ossabaw minipigs: Lean group – $n = 7$ (liver), $n = 1$ (abdominal SAT) and $n = 2$ (VAT). Obese group: $n = 4$ (liver), $n = 2$ (abdominal and neck SAT).

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et al., 2005), although no difference in gene expression was found between breeds. Another difference is that the diet of the Göttingen minipigs, in contrast to that of the other two breeds, did not contain high proportions of fat and/or sugar, which has previously been shown to result in adiposity and signs of metabolic syndrome (Dyson et al., 2006; Johansen et al., 2001; Lee et al., 2009). However, the Göttingen minipigs had been obese for a longer period of time than the other two breeds, which is closer to the situation in human obesity. In the domestic pigs, previous studies have shown that atherogenesis can be induced in domestic (Yorkshire) pigs following 4–12 weeks of feeding with excess fat and cholesterol (Gerrity et al., 1979, 2001). However, even though the domestic pigs in this study were fed a diet with excessive fat and sugar for more than 12 weeks (up to 6 months) the measured plasma indicators did not indicate atherogenesis in the obese pigs, except for the elevated total cholesterol levels (Christensen et al., 2012).

To our knowledge, no studies have compared expression of ORM in adipose tissue between obese and lean humans, therefore it was not possible to determine whether the unchanged adipose expression of ORM in obese compared to lean pigs is in agreement with human adipose tissue expression.

In summary, this investigation compares the similarities of three types of obese pig models (domestic, Göttingen and Ossabaw) and humans in terms of expression levels and serum levels of ORM. Our results, although obtained with a limited number of pigs provide evidence that only the Ossabaw minipig responds similarly to obesity as humans, with a higher serum ORM concentration in the obese pigs. This reinforces Ossabaw minipigs as a promising model for human obesity. As is the case in humans, no relationship between circulating ORM levels and its expression in either liver or adipose tissues was found in the three breeds. Furthermore, no differential expression of *ORM1* in the adipose tissues were found, as in obese humans (Alfadda et al., 2012). No studies have been made into the differential mRNA expression of ORM in lean and obese humans, however, studies in mice have shown an up-regulation in adipose tissues in obese compared to lean mice (Lee et al., 2010), and it would therefore be interesting to see if this is the case as well in humans.

Conflict of interest statement

The authors declare that no conflicting financial interests exist.

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6 Discussion

6.1 General discussion

In the domestic pigs, the RINs of the adipose tissues are generally slightly lower than that of the liver tissue, as are the 260/280 ratio and yields (ng/µl) (Table 5). In the Göttingen minipigs the quality of RNA varies somewhat between the tissues with the highest RINs in the liver (mean of 9) (Table 6). The RINs of the adipose tissues are all lower than the RINs of the liver, with averages of 5.5-7.1, which could have an impact on the results, especially if comparing liver and adipose tissue samples. Generally, the extractions of RNA from the adipose tissues proved to be more difficult than extractions from liver. This is due to several reasons: Firstly, the liver tissue has a higher density of cells as liver cells are smaller than adipocytes. When using the same amount of starting material (e.g. ½ g), the liver sample will contain a much higher amount of RNA than the adipose tissue sample. As the limitation for the methods used for RNA extraction is approximately 1 g this can have a high impact on the amount of RNA extracted from adipose tissue samples. Secondly, the lipid content of the adipose tissue appears to have an impact on the quality of RNA extracted from the adipose tissue. One of the steps of optimization described in section 2.1.1.1 was to remove the lipid content of the sample after homogenization, and this step improved the yield and quality of RNA greatly.

The variation within the methods of RNA extraction was tested by comparing the expression of the reference genes. In this way the 2 different methods for extracting RNA used in the adipose tissues of the domestic pigs were compared and no difference was found on the results. As described in section 2.1.1, the 260/230 ratio is used to estimate the contamination of organic compounds in a sample, and a ratio of 1.8-2 is accepted as a sample with no or little contamination. In all the studies in this thesis the ratio is very different within the tissues (Table 5, Table 6 and Table 7), however, when the expression of the reference genes were tested in samples having different 260/230 ratios within a tissue, this contamination did not have an impact on the expression of the reference genes, and was therefore not considered to be of importance for this assay.

Generally, extractions of RNA from the adipose tissues of the Ossabaw pigs were problematic, with low RINs as indicators of low quality RNA in especially the VAT (Table 7). It is possible that the samples were not snap frozen rapidly enough after tissue sampling or that the samples were not stored cold enough, so the RNA has become degraded as a consequence.

As described in the appendix p. 124 and 125, some samples were removed in the preprocessing of the data due to high variation between the technical replicates. For the domestic pigs, three samples were removed. The RINs of the three samples were within accepted range (6.9-8.9), and all had 260/230 and 260/280 ratios of about 2. Therefore, it would seem that variation introduced was not due to low quality RNA. No samples were removed from the dataset of the Göttingen minipigs, however, an entire cDNA-synthesis was removed due to low efficiency. Three samples were removed in the Ossabaw dataset due to high variation between technical replicates. These samples had low RINs, so the variation could be due to introduced variation or to low quality of RNA.

The fold change criterion used to point out biological relevant significantly differentially expressed genes was set to 1.5. During a normal, acute infection this number might be too low as most APPs and cytokines

show big changes in plasma levels during infections. For example, the APP CRP has an increase in plasma concentrations of up to 1000 times during infection in humans, and generally APPs are defined as plasma proteins whose concentrations increase by more than 2-fold following an inflammatory stimulus (Baumann & Gauldie 1990). However, the response to obesity-induced low-grade inflammation of cytokines and APPs is not as clear as during normal infections, and generally the plasma concentrations of these proteins are not raised to the same extent. For example, increases in CRP levels of <2 fold have been found to be associated with increased risk of cardiovascular disease in overweight women (Ridker *et al.* 1998), and IL-8 levels have been found to only increase by 1.3-fold in obese humans (Straczkowski *et al.* 2002). Therefore, it is important that the fold change criterion is set low enough to detect changes, but still high enough to make sure the changes are biologically relevant. Consequently, the fold change criterion was set to 1.5 for this work.

6.2 Study II

6.2.1 Göttingen minipigs

As the analysis of gene expression and blood proteins has already been discussed in Paper III p. 70, this section will only contain discussions on the data that has not been discussed elsewhere.

The hypothesis that the expression between the adipose tissues (Table 2 in Paper III p. 70) is differential, and that the adipose tissues from the abdomen are more metabolically active than neck SAT is confirmed. However, this is not just due to obesity as more genes are differentially regulated in the abdominal adipose tissues of the lean pigs than the obese. 17 out of 18 genes that are significantly differentially expressed in the neck SAT compared to the RPAT and VAT combined are expressed at lower levels in the neck SAT. In the obese pigs the same pattern is present, though with fewer genes being affected, as 10 out of 12 genes are downregulated in the neck SAT compared to the RPAT and VAT combined. Comparing RPAT and VAT in the lean pigs only seven genes are significantly differentially expressed and in the obese pigs nine genes are significantly differentially regulated between the RPAT and VAT. It is interesting that this is so, as it is expected that the differences in adipose tissue expression will increase in obesity, as the VAT and RPAT become more metabolically active during obesity than the neck SAT in humans (reviewed in (Clement & Langin 2007; Wronska & Kmiec 2012)). As the lean and obese pigs are of the same age, and has both been fed a standard diet, although in different quantities, it appears that obesity in itself with access to *ad libitum* feed actually lowers the differential response in all the investigated adipose tissues in the pigs. However, the obese pigs have been treated with therapeutic peptides in pharmacological studies at Novo Nordisk A/S, and it has not been possible to get any information on these peptides. Even though the pigs had been subjected to a wash-out period, it is possible that the unknown peptides could have an impact on the results of this study.

6.2.2 Ossabaw minipigs

There is a clear difference in the number of significantly differentially expressed genes between the lean and obese Ossabaw pigs in liver and the adipose tissues, as six genes are significantly differentially regulated in the liver, and only one is significantly differentially regulated in both investigated adipose tissues combined (Table 8). This could be due to the very low number of pigs in the groups of the adipose

tissues, making it difficult to achieve statistical power; in the liver the lean group consisted of seven pigs and the obese group consisted of five pigs. In the abdominal SAT, the lean and obese groups consisted of three pigs each, and in both the adipose tissues the data has high variation (SEM). One of the reasons for this could be the low quality of RNA extracted from the samples (Table 7) where the VAT samples showed consistently low RINs (mean of 2.7), and the samples from the abdominal SAT varied a great deal in RINs (RINs between 2.4 and 6.8), even though the 260/280 ratios were within accepted range (mean of 1.94). Another reason for the high SEMs could be that half the genes in the study showed DNA contamination/low specificity. This is surely to have an effect on the data. Unfortunately, it was not possible to extract RNA of higher quality, or without contamination, in these samples, even though numerous attempts to do so were carried out. The difference in RINs between the tissues could have an impact when comparing the gene expression between tissues, however, that analysis has not been performed in this study.

Of the genes investigated (Table 8) the genes showing significantly differential regulation all differ in regulation from expected, except *LBP* which is upregulated in the abdominal SAT. As shown in Table 1, the mRNA levels of *CCL2*, *FIB*, *HP*, *IL1RN* and *CD14* in the liver of humans are expected to rise in obesity, and the level of *TF* is expected to not be affected by obesity. Yet all the genes mentioned are downregulated in the liver of obese Ossabaw minipigs compared to lean. In the liver most genes show tendencies of down-regulation (20/26), however, in the adipose tissues most genes show tendencies of upregulation (19/25 in the abdominal SAT and 24/25 in the VAT). Even though this is only tendencies, and not statistically significant, it is interesting that obesity shifts the response of the innate immune genes from the liver to the adipose tissues. An acute inflammatory response will lead to hepatic secretion of several immune factors. However, as discussed, the low-grade inflammation caused by obesity is chronic, and not acute, and that could be the reason for this shift.

The serum concentrations of several proteins were not affected by obesity in the Ossabaw minipigs (Figure 12). There was no induction of TNF-α in either the lean or the obese minipigs, even though the expression of this inflammatory cytokine has been shown to be elevated in the serum of obese humans (Dandona *et al.* 1998). Over half the pigs of both groups had IL-6 serum concentrations below the detection limit of the ELISA, which is different than what has been found in obese humans (Khaodhiar *et al.* 2004). The expression of the remaining three serum proteins measured (CRP, HP and SAA) was not affected by obesity, which is also in contrast to what has been found in humans (Festa *et al.* 2001; Poitou *et al.* 2006; Chiellini *et al.* 2004). This could indicate that the pigs had not developed obesity-induced low-grade inflammation, even though the obese minipigs weighed significantly more than the lean. However, as shown in Table 10, the obese Ossabaw minipigs had higher levels of cholesterol (p=0.03) and triglycerides (p=0.02) in the blood than the lean minipigs, which indicate that the obese pigs are showing signs of metabolic perturbations, even if those perturbations are not yet evident in the innate immune factors in the blood.

6.3 Study III

A comparison between the 3 breeds of pig was difficult for all the reasons mentioned previously, such as the difference of age in the pigs, difference of diets, the genomic contamination of the samples from the Ossabaw minipigs and the differences in quality of RNA between the breeds and tissues. However, when investigating ORM, the NTC and –RT sample showed no contamination in either of the breeds, which indicates that, the *ORM1* primers were not affected by the contamination.

All breeds and tissues had samples that showed no *ORM1* expression as the *ORM1* levels were below detection level. Of the samples of the adipose tissues in the obese Göttingen minipigs, as well as the lean and obese Ossabaw minipigs, about half were removed due to expression below detection level (Paper IV p. 95, Figure 2). Therefore, it is difficult to know for sure whether it is the pigs with no *ORM1* expression or with *ORM1* expression that are outliers, as the normal response could be no expression in the adipose tissues of these pigs. To further investigate this, larger groups are needed to obtain statistical power.

When comparing lean and obese domestic pigs (Paper II p. 39, Figure 3A, C, E and G) with lean and obese Göttingen minipigs (Paper III p. 70, Figure 1A-D), it is clear that a higher number of genes in the Göttingen minipigs are affected by obesity, especially in the adipose tissues. In the adipose tissues of the domestic pigs 1-5 genes are differentially regulated in obesity, and in the Göttingen minipigs 8-12 genes are differentially regulated in obesity. This is despite the fact that the obese Göttingen minipigs were fed a standard diet and the domestic pigs were fed a high energy diet (Table 9) which is expected to have an impact on the metabolism of the pigs, and therefore on the obesity-response. However, the differences in the number of genes affected by obesity in the two breeds could be due to the difference in age between the breeds, as the domestic pigs are 7-9 months old and the Göttingen minipigs are 41-47 months.

More genes are differentially regulated in the RPAT of the Göttingen minipigs than in the abdominal SAT of the domestic pigs (Paper III p. 70, Figure 1B and Paper II p. 39, Figure 3C, respectively). The RPAT is part of the intra-abdominal adipose tissue as is the VAT, and differs from abdominal SAT in some respects, such as having a higher lipid uptake (Mårin *et al.* 1992). In other regards, RPAT is more similar to abdominal SAT than VAT, for example in correlations between lipid uptake and fasting insulin as well as fasting glucose (Mårin *et al.* 1992), and RPAT has been shown to be drained by the inferior vena cava, as does abdominal SAT, where VAT is drained by the portal vein (Björntorp 1990; Abate *et al.* 1994). The drainage into the portal vein exposes liver cells directly to adipokines secreted from the VAT and this has been associated with systemic inflammation in obese humans (Fontana *et al.* 2007). For these reasons the abdominal SAT and RPAT have been grouped together in comparisons between the Göttingen minipigs and the other pigs.

The Göttingen minipigs and the domestic pigs show the same response to obesity in the regulation of gene expression between the tissues (Paper II p. 39, Table 2 and Paper III p. 70, Table 2), as both breeds exhibit a lower number of differentially regulated genes between the abdominal adipose tissues (RPAT/abdominal SAT and VAT) and the neck SAT in the obese pigs compared to lean. A total of 16 genes are differentially regulated in the abdominal adipose tissues in the lean domestic pigs and Göttingen minipigs compared to neck SAT, where 12 and nine genes are differentially regulated in the abdominal adipose tissues in the obese Göttingen minipigs and domestic pigs, respectively.

7 Conclusions and perspectives

Based on the results presented in this thesis it is not possible to determine a completely human-like innate immune response to obesity in any of the three breeds of porcine obesity-models studied.

Cloning did not reduce the inter-individual phenotypic variation, as variability was found to be the same between the cloned and control domestic pigs, in all 4 tissues, and in serum proteins, except for the protein ORM in both the lean and obese clones.

Obesity altered the expression of the innate immune genes, as fewer genes were differentially regulated in the obese controls and clones as compared to the lean controls and clones. Furthermore, cloning induced a differentiated response to obesity in innate immune genes in liver and three types of adipose tissue as fewer genes were differentially regulated in the lean and obese clones than in the lean and obese controls. The effect were additive, making the obese clones the group with fewest differentially regulated genes in the four tissues investigated. However, two of the measured serum proteins showed increased concentrations in the obese cloned pigs compared to the lean cloned pigs, and the protein concentrations were unchanged between the lean and obese control pigs.

The abdominal tissue from the abdomen; abdominal SAT and VAT, did not show a higher number of affected innate immune response genes than the neck SAT, except in the VAT of the obese clones.

As a method to reduce group sizes of animal models, by lowering the inter-individual phenotypic variation, cloning may not be the way forward. The results from this thesis is supported by other investigations into the variation between the cloned pigs having shown that cloned pigs does not show less inter-individual phenotypic variation than controls (Clausen *et al.* 2011; Hwang *et al.* 2009; Park *et al.* 2011; Whyte *et al.* 2011). Furthermore, other studies have shown deviant phenotypes of cloned animals (Clausen *et al.* 2011; Christensen *et al.* 2012; Whyte *et al.* 2011; Park *et al.* 2011; Archer *et al.* 2003; Jiang *et al.* 2007). Coupled with the low efficiency of the method, with increased mortality perinatally, the method is time-consuming and expensive, and perhaps it is time to focus on other animal models until the method has been improved.

Even though the Göttingen minipigs show a varied differential response to obesity in all tissues, the response does not translate to the serum protein expression, which is not as expected and different from the human responses to obesity. This could possibly be due to the feed, which was not high calorie feed, but a standard chow fed *ad libitum*. Because of the lack of differential expression in serum proteins, it is not possible, with the standard chow, to establish the Göttingen minipig as a suitable model for human obesity-induced innate immune responses. However, it is possible that a different feed with high calories or that using pigs which had not been subjected to pharmacological treatments, would change this conclusion.

The metabolic activity of the neck SAT was found to be lower than that of the other two adipose tissues in especially the lean Göttingen minipigs. However, the difference in differential expression was expected to increase in obesity, which is not the case for these pigs. Therefore the conclusion in regards to this must be that the access to *ad libitum* feeding of a standard diet could have a positive effect on the effects of obesity-induced low-grade inflammation.

The Ossabaw minipigs show little response to obesity in both gene and serum protein expression, even though blood parameters and body weights show signs of metabolic perturbations. As the lack of response

is very different from what has been found in humans, it is not possible to establish the Ossabaw minipig as a suitable model for human obesity-induced innate immune responses. As the results of this study are limited due to small group sizes it would be very interesting to obtain more samples to enlarge the group sizes and to hopefully obtain RNA of higher quality, and to repeat the investigations.

The response of the Göttingen minipigs is more human-like than the response of the domestic pigs in terms of the gene expression of the four tissues. In terms of the serum protein expression, the Ossabaw minipigs shows a more human-like response than the other two breeds as it shows an elevated concentration of the APP ORM, which has been shown to be elevated in obese humans.

The gene expression of *ORM1* in the liver and three adipose tissues was unchanged between the lean and obese pigs in all three breeds, although bigger groups of the Göttingen and Ossabaw minipigs will be needed to confirm this result. It would be of great interest to investigate whether humans exhibit a differential expression of *ORM1* in the adipose tissues in obesity, as has been found in mice (Lee *et al.* 2010). It is possible, even if the response of rodent obesity models to obesity has been found to be different from humans, that the differential expression of *ORM1* in the adipose tissues is more similar to the expression of mice than the expression of pigs.

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9 Appendix

9.1 Preprocessing steps – Study I

Using Fluidigm Real-Time PCR Analysis software (Fluidigm, CA, USA), GeNorm software (Vandesompele et al, 2002), and GenEx5 software (MultiD, Göteborg, Sweden).

1. Check efficiency for all primers (between 0.8-1.1 is accepted)
2. Check correlation for all primers (>0.98 is accepted)
    i. Should have been more diluted: FIB, HP and ORM1
    ii. Problems with serial dilution (efficiency set to 100%): IL8(b), IL1b, IL6, IL8(a), TNF in liver and IL10(b) in adipose tissues
    iii. Due to low efficiencies MUC1, DEFB1 and IL12 is removed
3. Check melting curves (differences inside 1°C is accepted)
4. Interrun calibrate
5. Cutoff values off scale (Cq value >26 – due to primer dimer)
6. Check for missing values; if less than 15% missing values pr primer, replace with max Cq+2
    i. Removed due to >15% missing samples: SPA1 from abdominal SAT and neck SAT, and LBP from VAT.
7. Correct data for efficiency for each primer set
8. Select reference genes on the basis of evaluation using GeNorm
    i. Liver: HPRT1 and GAPDH – M=0.3
    ii. Adipose tissues: HPRT1 and B2M – M=0.55
    iii. All tissues combined: RPL13A and GAPDH – M=0.8
9. Normalize data with geometric means from reference genes; liver, adipose tissues and combined
10. Check standard deviation of the technical replicates (variation of more than 1 cq value is removed)
    i. The samples: If variation between the technical replicates is >30% the samples are removed: Tissues combined; an obese clone from Group 1 is removed from liver (9601), a lean control from Group 3 is removed from VAT (3149), and a lean clone is removed from neck SAT (3297). Single tissues; a lean clone from Group 3 is removed from neck SAT (3297).
    ii. The genes: If variation between the technical replicates is >15% the genes are removed: Tissues combined; IL8(b) and IL6 in liver, FIB, IFNG, LBP, IL1RN and ORM1 in adipose tissues. Single tissues; IL8(b) and IL6 in liver, FIB, IFNG, LBP, IL1RN, ORM1 and IL10(b).
11. Average technical replicates
12. Convert Cq values to relative quantities; lowest number (highest expression) is set to 1
13. Log2 transform data

9.2 Preprocessing steps – Study II

Using Fluidigm Real-Time PCR Analysis software (Fluidigm, CA, USA), GeNorm software (Vandesompele et al, 2002), and GenEx5 software (MultiD, Göteborg, Sweden).

1. Check efficiency for all primers (between 0.8-1.1 is accepted)
2. Check correlation for all primers (>0.98 is accepted)
    i. Should have been more diluted: TNF and C3
3. Check melting curves (differences inside 1°C is accepted)
4. Interrun calibrate
5. Cutoff values off scale (Cq value >26 – due to primer dimer)
6. Check for missing values; if less than 15% missing values pr primer, replace with max Cq+2
    i. Removed due to >15% missing samples: Both breeds, adipose tissues; LBP, AOAH, SPA1, ORM1(a) and ORM1(b), Göttingen adipose tissues; SPA1 and ORM1(a), both breeds, liver: ADIPOQ
7. Correct data for efficiency for each primer set
8. Select reference genes on the basis of evaluation using GeNorm
    i. Both breeds combined, adipose tissue: RPL13A and GAPDH – M=0.96
    ii. Both breeds combined, liver: RPL13A and GAPDH – M=0.55
    iii. Göttingen, adipose tissues: GAPDH, B2M, RPL13A, ACTB and HPRT1 – M=1.26
    iv. Göttingen, liver: GAPDH, B2M, RPL13A and ACTB – M=0.6
    v. Göttingen, all tissues combined: RPL13A and ACTB – M=0.62
    vi. Ossabaw, adipose tissues: GAPDH – M=1.05
    vii. Ossabaw, liver: GAPDH, B2M and RPL13A – M=0.55
    viii. Ossabaw, all tissues combined: RPL13A and GAPDH – M=1.43
9. Normalize data with geometric means from reference genes: for both breeds; liver, adipose tissues and combined, as well as both breeds combined
10. Check standard deviation of the technical replicates (variation of more than 1 cq value is removed)
    i. The samples: If variation between the technical replicates is >30% the samples are removed: Ossabaw all tissues combined – two lean pigs was removed from VAT (1390 and 1371); Ossabaw adipose tissues – 2 lean pigs were removed from VAT (1390 and 1371) and a lean pig from abdominal SAT (1393); Göttingen and Ossabaw adipose tissues combined – three lean pigs from VAT was removed (1390, 1371 and 1393)
    ii. The genes: If variation between the technical replicates is >15% the genes are removed: Göttingen and Ossabaw liver- IL6(a), IL8(a), IFNG, AOAH, TNF, SPA1 and ORM1(b); Göttingen and Ossabaw adipose tissues – FIB, IL1B and IFNG; Ossabaw all tissues combined – CRP, IL1B, IL6(a), SPA1, COX2, IL6(B), IL8(a), ITIH4(b), IL18, ORM1(a), IL10(b), ADIPOQ, IFNG, TNF, AOAH, CD36 and ORM(b); Ossabaw adipose tissues – CD40, CRP, DEF2, FIB, IL10(b), IL1B, ITIH4(b), IL18, IFNG, TNF, ORM1(b), TLR4, AOAH, CXCL10, CCL3, COX2 and IL6(b); Göttingen all tissues combined – IL1B, IL8(a), AOAH, SPA1, ORM1(a), ORM1(b), ADIPOQ and LBP; Göttingen adipose tissue (accepted variation set to 22%, as SD was high due to a cDNA-synthesis with low

efficiency that was removed) – FIB, IL8(b), IL1B, IL8(a), IFNG, AOAH, IL10(b) and ORM1(b).

11. Average technical replicates
12. Convert Cq values to relative quantities; lowest number (highest expression) is set to 1
13. Log2 transform data

9.3 Purification of RNA from non-adipose tissues

Materials:

RNase-Free DNase set (Qiagen, #79254)
RNeasy Lipid Tissue Midi (Qiagen, #75842)
QIAzol Lysis Reagent (200 ml) (Qiagen #79306)
Chloroform (Merck #1.02445.1000)
M Tubes for GentleMACS (Fisher #mil-130-093-458)

Method:

- Approximately ½ g of tissue is transferred to a M Tube with 5 mL Qiazol Lysis Reagent. Homogenize sample on GentleMACs dissociater, on program: 02.01 Frozen M.Tube
- Transfer homogenized sample to nunc tube supplied in the kit, and place the sample at room temperature (15-25° C) for 5 minutes.
- Add 1 mL chloroform (0,2 mL pr 1 mL QIAzol Lysis Reagent). Shake the tube vigorously for 15 seconds, and place the sample at room temperature for 2-3 minutes.
- Centrifuge tubes for 15 minutes at 4500 rpm at 4° C.
- Homogenate is now divided into three phases. Transfer approximately 2.75 mL from the upper phase to a new 15 mL nunc tube, and add approximately 2.75 mL of 70% ethanol (1:1). Mix homogenate with the pipette.
- Transfer up to 4 ml of homogenate to a RNeasy Maxi Spin Column. Centrifuge tubes for 5 minutes at 4500 rpm at 20-25° C. Discard flow-through. Repeat step and discard flow-through.
- Transfer 2 mL Buffer RW1 to column and centrifuge for 5 minutes at 4500 rpm at 20-25° C. Discard flow-through.
- Mix DNase I Incubation mix: 20 µL of DNase I Stock Solution is added to 140 µL Buffer RDD pr tube. Mix carefully with the pipette. Transfer 160 µL DNase I Stock Solution to the RNeasy silica-gel membrane and place at room temperature for a minimum of 15 minutes.
- Add 2 mL Buffer RW1 to the column and centrifuge for 5 minutes at 4500 rpm at 20-25° C.
- Add 2.5 mL Buffer RPE to the column and centrifuge for 5 at 4500 rpm at 20-25° C. Discard flow-through. Repeat step. The column is now ethanol-free.
- Transfer column to new nunc tube, and add 150 µl RNase-free water to the membrane. Place at room temperature for 1 minute and centrifuge for 3 minutes at 4500 rpm at 20-25° C.
- Re-use flow-through on the membrane and centrifuge for 5 minutes at 4500 rpm at 20-25° C.
- Add further 150 µL RNase-free water to the membrane and centrifuge for 5 minutes at 4500 rpm at 20-25° C.
- Measure concentration on Nanodrop.

9.4 Purification of RNA from adipose tissues (domestic pig)

Materials:

RNase-Free DNase set (Qiagen, #79254)
RNeasy Maxi kit (Qiagen #75162)
QIAzol Lysis Reagent (200 ml) (Qiagen #79306)
Chloroform (Merck #1.02445.1000)
M Tubes for GentleMACS (Fisher #mil-130-093-458)

Method:

- Approximately 1 g of tissue is transferred to a M Tube with 10 mL Qiazol Lysis Reagent. Homogenize sample on GentleMACs dissociater, on program: 02.01 Frozen M.Tube
- Transfer homogenized sample to nunc tube supplied in the kit, and place the sample at room temperature (15-25° C) for 5 minutes.
- Add 4 mL chloroform (0,2 mL pr 1 mL QIAzol Lysis Reagent). Shake the tube vigorously for 15 seconds, and place the sample at room temperature for 2-3 minutes.
- Centrifuge tubes for 15 minutes at 4500 rpm at 4° C.
- Homogenate is now divided into three phases. Transfer approximately 11 mL from the upper phase to a new 50 mL nunc tube, and add approximately 11 mL of 70% ethanol (1:1). Mix homogenate with the pipette.
- Transfer up to 15 ml of homogenate to a RNeasy Maxi Spin Column. Centrifuge tubes for 5 minutes at 4500 rpm at 20-25° C. Discard flow-through. Repeat step and discard flow-through.
- Transfer 7.5 mL Buffer RW1 to column and centrifuge for 5 minutes at 4500 rpm at 20-25° C. Discard flow-through.
- Mix DNase I Incubation mix: 30 µL of DNase I Stock solution is added to 210 µL Buffer RDD pr tube. Mix carefully with the pipette. Transfer 240 µl DNase I Stock Solution to the RNeasy silica-gel membrane and place tube at room temperature for a minimum of 15 minutes.
- Add 7.5 mL Buffer RW1 to the column and centrifuge for 5 minutes at 4500 rpm at 20-25° C. Discard flow-through. Repeat step.
- Add 10 mL Buffer RPE to the column and centrifuge for 2 minutes at 4500 rpm at 20-25° C. Discard flow-through.
- Add another 10 mL Buffer RPE to the column and centrifuge for 10 minutes at 4500 rpm at 20-25° C. Discard flow-through.
- Transfer column to new nunc-tube and add 800 µL RNase-free water to the membrane. Place tube at room temperature for 10 minutes and centrifuge at 4500 rpm at 20-25° C.
- Re-use flow-through on the membrane and centrifuge for 5 minutes at 4500 rpm at 20-25° C.
- Measure concentration on Nanodrop.

9.5 Purification of RNA from adipose tissues (optimized - domestic pig)

Materials:

QIAzol Lysis Reagent (200 ml) (Qiagen #79306)
Isopropanol (2-propanol, Merck #1.09634.1000)
Chloroform (Merck #1.02445.1000)
M Tubes for GentleMACS (Fisher #mil-130-093-458)

Method:

- Approximately ½ g of tissue is transferred to a M Tube with 10 mL Qiazol Lysis Reagent. Homogenize sample on GentleMACs dissociater, on program: 02.01 Frozen M.Tube
- Transfer homogenized sample to a 15 mL nunc tube, and place the sample at room temperature (15-25° C) for 5 minutes.
- Add 2 mL chloroform (0,2 mL pr 1 mL QIAzol Lysis Reagent). Shake the tube vigorously for 15 seconds, and place the sample at room temperature for 2-3 minutes.
- Centrifuge tubes for 20 minutes at 4500 rpm at 4° C.
- Homogenate is now divided into three phases. Transfer approximately 2.2 mL from the upper phase to a new nunc tube, and add 1.4 mL of Isopropanol (1:0.625). Place the tube at room temperature for 5 minutes. Centrifuge tubes for 10 minutes at 4500 rpm at 4° C.
- Aspirate carefully and discard the supernatant.
- Washing step: Add 10 mL of cold 75% ethanol (1 mL pr 1 mL Lysis Reagent) and make sure the pellet is properly washed. Centrifuge for 5 minutes at 3300 rpm at 4° C.
- Repeat the washing step 2 times, with 5 ml of cold 75% ethanol. Discard the supernatant between each washing step.
- Remove the supernatant completely and let the RNA pellet air dry for 30 minutes.
- Dissolve pellet in 100 µL RNase-free water, place sample at room temperature for 5-10minutes and then vortex.
- Measure concentration on Nanodrop.

9.6 Purification of RNA from adipose tissues (optimized - Göttingen and Ossabaw minipigs)

Materials:

QIAzol Lysis Reagent (200 ml) (Qiagen #79306)
Isopropanol (2-propanol, Merck #1.09634.1000)
Chloroform (Merck #1.02445.1000)
M Tubes for GentleMACS (Fisher #mil-130-093-458)

Method:

- Approximately ½ g of tissue is transferred to a M Tube with 4 mL Qiazol Lysis Reagent. Homogenize sample on GentleMACs dissociater, on program: 02.01 Frozen M.Tube
- Transfer homogenized sample to two 2 mL eppendorf tubes, and place the sample at room temperature (15-25° C) for 5 minutes.
- Centrifuge the tubes for 10 minutes at 12000xg at 4° C. Transfer the pink phase to new eppendorf tubes (avoid pellet and fatty layer on top)
- Add 400 µL chloroform (0.2 mL pr 1 mL QIAzol Lysis Reagent). Shake the tube vigorously for 15 seconds, and place the sample at room temperature for 3 minutes.
- Centrifuge tubes for 15 minutes at 12000xg at 4° C.
- Homogenate is now divided into three phases. Transfer approximately 900 µL from the upper phase to a new eppendorf tube, and add 1 mL of Isopropanol. Vortex and place the tube at room temperature for 5 minutes. Centrifuge tubes for 10 minutes at 12000xg at 4-25° C.
- Aspirate carefully and discard the supernatant.
- Wash the sample 2 times: Add 1 mL of cold 75% ethanol and vortex to make sure the sample is properly washed. Centrifuge for 8 minutes at 8000xg at 4-25° C. Discard the supernatant between each washing step.
- Remove the supernatant completely and dry the RNA pellet in a thermoblock at 55° C for 2 minutes.
- Dissolve pellet in one of the tubes in RNase-free water (30-100 µl, depending on the size of pellet), and mix. Transfer the water to the other tube and mix.
- Place tube on thermoblock at 55° C for 10 minutes to dissolve the pellet completely.
- Measure concentration on Nanodrop.

9.7 cDNA-synthesis

Materials

QuantiTect Reverse Transcription Kit (Qiagen #205311)

Methods

- Add 2 µl gDNA Wipeour Buffer (7x) to a 0.2 mL mini-eppendorf tube. Add a mix of 12 µL RNase-free water and RNA (final concentration 500 ng total RNA), to a total of 14 µL
- Incubate in Thermocycler (Qiagen) for 2 minutes at 42°C -> 4°C
- Make Master mix for all samples (+10%) :
    - For 1 reaction : 1 µL Reverse Transcriptase

      1 µL RT Primer Mix

      4 µL RT buffer
- Add 6 µL Master mix to each tube with RNA
- Incubate in Thermocycler for 15 minutes at 42°C -> 3 minutes at 95°C -> 4°C
- The 20 µL of cDNA can be stored at -20°C until use
- For Fluidigm: measure cDNA concentrations on the NanoDrop and dilute cDNA to approximately 200 ng/µL in low EDTA TE-buffer before preamplification (all samples are diluted with the same amount of low EDTA TE-buffer)

9.8 qPCR with Rotor-Gene Q

Materials

Sybr Green Jumpstart Taq Readymix without $MgCl_2$ (Sigma # S5193)

Method

- Make the dilution series:

	Koncentration (µg/5µl)	cDNA (µl)	H2O (µl)
1	0,667	6	39
2	0,222	15 (fra 1)	30
3	0,0741	15 (fra 2)	30
4	0,0247	15 (fra 3)	30
5	0,00823	15 (fra 4)	30
6	0,00274	15 (fra 5)	30
7	0,000914	15 (fra 6)	30
8	0,000305	15 (fra 7)	30

	Koncentration (µg/5µl)	cDNA (µl)	H2O (µl)
1	10	30	0
2	1	3 (fra 1)	27
3	0,1	3 (fra 2)	27
4	0,01	3 (fra 3)	27
5	0,001	3 (fra 4)	27
6	0,0001	3 (fra 5)	27
7	0,00001	3 (fra 6)	27
8	0,000001	3 (fra 7)	27

- Dilute primers: 10 µl F + 10 µl R + 180 µl H_2O => 10 µM (final concentration – 300 µM)
- Make the Mix according to table over qPCR-setup
- Add 20 µL Mix to each 0.1 mL mini-eppendorf tube (end with NTC)
- Add 5 µL of sample cDNA to each 0.1 mL mini-eppendorf tube and 5 µL RNase-free water in the NTC
- Place on the Rotor-Gene Q Instrument

9.9 Preamplification before Fluidigm

Materials

Man PreAmp Master Mix (Applied Biosystems, PN 4391128)
Low EDTA TE-buffer (VWR, APLIA8569.0500)

Method

- Prepare primer mix (max 100 primers), all primers are shaken and centrifuged before use
    - Make 20 µM-dilution of all primer pars (10 µL F primer (100 µM) + 10 µL R primer (100 µM) + 30 µl low EDTA TE-buffer)
    - Primer Mix: Mix 5 µL 20 µM primer-dilution from all primers and add low EDTA TE-buffer for a final dilution of 500 µL (200 nM).
- Defrost the cDNA and vortex briefly
- Prepare the PreAmp mix:
    - For 1 reaction: 5 µL TagMan PreAmp Master Mix + 200 nM primer mix
- Add 7.5 µL PreAmp mix to 0.2 mL mini-eppendorf tubes, and add 2.5 µL diluted cDNA
- Incubate in Thermocycler for 10 minutes at 95°C -> 15 seconds at 95°C -> 4 minutes at 60°C for 16 cycles -> 4°C
- Dilute preamplified cDNA in 30 µL low EDTA TE-buffer and store at -20°C (undiluted preamplified cDNA are used for dilution series)

9.10 qPCR with Fluidigm (EvaGreen)

Materials

TaqMan Gene Expression Master mix (Applied Biosystems, PN 4369016)
EvaGreen 20X (VWR, BTIU31000)
Low EDTA TE-Buffer (VWR, APLIA8569.0500)
BMK-M-48.48 B (Fluidigm)
85000800 sample and assay loading kit (Fluidigm), 100-0388 DNA binding Dye (Fluidigm)

Method

- Turn on the MX IFC controller
- Make Assay Master mix:

Assay mastermix	x 1	x 60
2X Assay loading Reagent	2.5 µl	150 µl
1X low EDTA TE-Buffer	0.25 µl	15 µl

- Dispense 2.8 µL Assay Master mix (6x8) into a microtiter-plate, and then add 2.3 µL 20 µM (final volume 5.1 µL) of specific primer pars A1-6, B7-12, C13-18 and so forth. Put film on plate and place in refrigerator until use.
- Make Pre-sample mix:

Pre-sample mix	x 1	x 60
2X TaqMan Gene Expression Mastermix	3 µl	180 µl
20X DNA binding Dye	0.3 µl	18 µl
EvaGreen 20X	0.3 µl	18 µl
1 x low EDTA TE-Buffer	0.9 µl	54 µl

- Dispense 4.6 µL Pre-sample mix (6x8) to another microtiter-plate (to avoid contamination), and add 1.5 µL preamplified cDNA to all wells. Put film on plate and place in refrigerator until use.
- Add Control Line Fluid to the chip.
- Remove protection-film from chip (is saved) and prepare the chip in the MX IFC Controller on the Chip Prime 113x-program (11 minutes). The chip is to be used within an hour of priming.
- Place the chip with the severed corner on the top left. The two microtiter-plates are vortexed and centrifuged at 1000 rpm for 30 seconds at 21°C.
- 4.9 µL of the Assay mix is added to the left side of the chip, and 4.9 µL of the sample mix is added to the right side of the chip.
- Checks for bobbles!
- The chip is placed in the MX IFC controller on the Load Mix 113x-program (1 hour).
- Turn on BioMark, turn on computer and start “data collection” and “turn on lamp”.

- After loading and mixing remove dust from the surface of the crip with tape and place the chip in the BioMark. Start new run with the following parameters:
    - Application type: Gene expression
    - Passive ref: ROX
    - Single probe
    - Probe type: EvaGreen
    - Thermal cycling protocol: 48x48 qPCR (10 min…)
- When the run has completed, the file is opened in the Chiprun Extractor and is divided into a PCR cycle file and a melt curve file

9.11 Detailed composition of experimental diets

Domestic pigs

Feed composition

Ingredients	%
Wheat flour	48.0
Wheat bran	16.7
Sugar	10.0
Vegetable fat	10.0
Casein	8.8
Potato protein concentrate	2.0
Soy protein concentrate	2.0
Vitamins/minerals	2.5
Calculated nutrient content	
Protein	17.1
Fat	11.6
Sugar	10.0
Dietary fibre	9.0
Energy (%)	
Protein	18
Fat	25
Carbohydrate	57

Information of the diet is from (Christensen *et al.* 2012), where further information of the content of vitamins and minerals in the diet can be found.

Ossabaw minipigs

	Control Chow (n = 15)	Atherogenic Diet Group (n = 13)
Average energy intake (kcal/day)	2500	6000
Carbohydrates (%)*	71	43
Starch (%)	41.6	25.2
Sucrose (%)	1.5	0.9
Fructose (%)	0.5	20
Glucose (%)	0.4	0.2
Protein (%)†	18.5	8.0
Fat (%)† fat source	10.5	46.0
	Hydrogenated soybean oil	Hydrogenated soybean oil
Cholesterol	Nil to negligible	2% by weight
Sodium Cholate	Nil to negligible	0.7% by weight
Methionine (ppm)	3500	2100
Choline (ppm)	1500	900

* Values represent percent of total daily calories.

Information of the diets are from (Lee *et al.* 2009).

Göttingen minipigs

Altromin 9023 (lean pigs)

Rohnährstoffe | Crude Nutrients	[%]	Mineralstoffe | Minerals	[%]
Trockensubstanz | Dry Matter	88.0	Calcium	1.0
Rohprotein | Crude Protein	13.5	Phosphor | Phosphorus	0.7
Rohfett | Crude Fat	2.5	Magnesium	0.3
Rohfaser | Crude Fibre	13.0	Natrium | Sodium	0.2
Rohasche | Crude Ash	7.5	Kalium | Potassium	0.6
NfE Stickstoff freie Extraktstoffe | Nitrogen Free Extracts	51.5		

Umsetzbare Energie | Metabolizable Energy	10.5 MJ/kg (2509 kcal/kg)
Kalorien aus Protein | Calories from protein*	19 %
Kalorien aus Fett | Calories from fat*	8 %
Kalorien aus Kohlenhydraten | Calories from carbohydrates*	73 %

(* berechnet mittels Atwater Faktoren | calculated using Atwater factors)

Altromin 9033 (obese pigs)

Rohnährstoffe | Crude Nutrients	[%]	Mineralstoffe | Minerals	[%]
Trockensubstanz | Dry Matter	88.0	Calcium	0.9
Rohprotein | Crude Protein	20.5	Phosphor | Phosphorus	0.6
Rohfett | Crude Fat	4.0	Magnesium	0.2
Rohfaser | Crude Fibre	5.0	Natrium | Sodium	0.2
Rohasche | Crude Ash	6.0	Kalium | Potassium	1.0
NfE Stickstoff freie Extraktstoffe | Nitrogen Free Extracts	52.5		

Umsetzbare Energie | Metabolizable Energy	12.1 MJ/kg (2891 kcal/kg)
Kalorien aus Protein | Calories from protein*	25 %
Kalorien aus Fett | Calories from fat*	11 %
Kalorien aus Kohlenhydraten | Calories from carbohydrates*	64 %

(* berechnet mittels Atwater Faktoren | calculated using Atwater factors)

Information is from altromin.com, where further information of the content of vitamins and minerals in the diet can be found.

9.12 Biochemical characteristics of domestic pigs

Table 2. Biochemical Characteristics of Clone and Control Pigs at 8 and 1/2 Months of Age; Fasting Values (Mean ± SEM)[a]

	ad libitum		diet restricted		*P* values[b]		
	clone (n = 9)	control (n = 10)	clone (n = 8)	control (n = 9)	type[c]	diet[d]	type × diet
feed intake[e] (kg DM/d)	2.48 ± 0.13b	2.97 ± 0.16a	1.37c	1.37c	<0.001	<0.001	0.18
energy intake[f] (MJ/d)	45.8 ± 2.2b	54.8 ± 2.7a	25.3c	25.3c	<0.001	<0.001	0.18
daily weight gain[g] (kg)	0.89 ± 0.04b	1.11 ± 0.03a	0.45 ± 0.03c	0.37 ± 0.01d	<0.001	<0.001	<0.001
glucose (mM)	5.08 ± 0.28a	4.39 ± 0.16b	4.61 ± 0.10b	4.50 ± 0.09b	<0.001	0.76	0.59
cholesterol (mM)	2.29 ± 0.05b	3.18 ± 0.14a	2.01 ± 0.05c	2.06 ± 0.07bc	<0.001	0.09	0.01
HDL (mM)	0.78 ± 0.06b	1.07 ± 0.05a	0.81 ± 0.02b	0.90 ± 0.05b	<0.001	0.28	0.06
LDL (mM)	1.24 ± 0.04b	1.74 ± 0.09a	0.83 ± 0.04c	0.82 ± 0.04c	<0.001	0.01	0.05
LDL/HDL	1.70 ± 0.62a	1.66 ± 0.36a	1.03 ± 0.12b	0.94 ± 0.23b	0.66	<0.001	0.94
triglyceride (mM)	0.43 ± 0.03	0.40 ± 0.05	0.26 ± 0.02	0.27 ± 0.03	0.54	0.13	0.85
NEFA[h] (μ ekv./L)	169.7 ± 47.1b	162.8 ± 32.7b	304.2 ± 33.2a	300.1 ± 22.0a	0.84	0.02	0.91
lactate (mM)	6.23 ± 0.79a	2.52 ± 0.44b	2.08 ± 0.48b	1.42 ± 0.31b	<0.001	0.10	0.05
insulin (ng/mL)	0.14 ± 0.07	0.17 ± 0.03	0.03 ± 0.01	0.07 ± 0.02	0.71	0.15	0.87
IGF-1[I] (ng/mL)	113.4 ± 13.2b	200.6 ± 19.9a	55.2 ± 9.2c	123.8 ± 14.3b	<0.001	0.11	0.82

[a]Means with the same letter are not significantly different. [b]Linear mixed model. [c]Type refers to clones and controls. [d]Diet refers to ad libitum feeding or diet restriction. [e]The pigs were given 1.5 kg of feed when diet restricted of which they ate all. [f]Calculated from metabolizable energy in the diet (Table 1) and feed intake. [g]Estimated by linear regression. [h]NEFA: nonesterified fatty acids. [I]IGF-1: insulin-like growth factor 1.

The table of characteristics of the domestic pigs are from (Christensen *et al.* 2012).